SHOUT
SHARE
IT

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

DANIEL L. ALKON, National Institutes of Health and Marine Biological Laboratory
FREDERICK B. BANG, Johns Hopkins University
EDWARD M. BERGER, Dartmouth College
STEVEN C. BROWN, State University of New York at Albany
HARLYN O. HALVORSON, Brandeis University
J. B. JENNINGS, University of Leeds
MEREDITH L. JONES, Smithsonian Institution
GEORGE O. MACKIE, University of Victoria
JOEL L. ROSENBAUM, Yale University
HOWARD A. SCHNEIDERMAN, Monsanto Company
F. JOHN VERNBERG, University of South Carolina
E. O. WILSON, Harvard University

DR. CHARLES B. METZ, University of Miami
Managing Editor

VOLUME 159

AUGUST TO DECEMBER, 1980

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

The Biological Bulletin is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $10.00. Subscription per volume (three issues), $27.00.

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and to Dr. Charles B. Metz, Institute For Molecular and Cellular Evolution, University of Miami, 521 Anastasia, Coral Gables, Florida 33134 during the remainder of the year.

The Biological Bulletin (ISSN 0006–3185)

Second-class-postage paid at Woods Hole, Mass., and additional mailing offices.

LANCASTER PRESS, INC., LANCASTER, PA.

CONTENTS

No. 1, August 1980

Annual Report of the Marine Biological Laboratory 1

Arnold, John M., and Lois D. Williams-Arnold
Development of the ciliature pattern on the embryo of the squid, *Loligo pealei:* A scanning electron microscope study 102

Bigger, Charles H.
Interspecific and intraspecific acrorhagial aggressive behavior among sea anemones: A recognition of self and not-self 117

Hatch, Walter I.
The implication of carbonic anhydrase in the physiological mechanism of penetration of carbonate substrata by the marine burrowing sponge *Cliona celata* (Demospongiae) 135

Hildreth, Jane E., and William B. Stickle
The effects of temperature and salinity on the osmotic composition of the southern oyster drill, *Thais haemastoma* 148

Nelson, Keith, Dennis Hedgecock, Will Borgeson, Eric Johnson, Richard Daggett, and Diane Aronstein
Density-dependent growth inhibition in lobsters, *Homarus* (Decapoda, Nephropidae) 162

Seigfried, Clifford A.
Seasonal abundance and distribution of *Crangon franciscorum* and *Palaemon macrodactylus* (Decapoda, Caridae) in the San Francisco Bay-Delta 177

Seigfried, Clifford A., and Mark E. Kopache
Feeding of *Neomysis mercedis* (Holmes) 193

Steel, C. G. H.
Mechanisms of coordination between moulting and reproduction in terrestrial isopod Crustacea 206

Sugimoto, Keiji, and Hiroshi Watanabe
Studies on reproduction in the compound ascidian, *Symplegma reptans:* Relationship between neural complex and reproduction 219

Warner, Jon A., and James F. Case
The zoogeography and dietary induction of bioluminescence in the midshipman fish, *Porichthys notatus* 231

Wickham, Daniel E.
Aspects of the life history of *Carcinonemertes errans* (Nemertea: Carcinonemertidae), an egg predator of the crab *Cancer magister* 247

No. 2, October 1980

Anderson, Robert S.
Hemolysins and hemagglutinins in the coelomic fluid of a polychaete annelid, *Glycera dibranchiata* 259

HADLOCK, ROBIN P.
Alarm response of the intertidal snail *Littorina littorea* (L.) to predation by the crab *Carcinus maenas* (L.) 269
HOUK, MARGARET S. AND RALPH T. HINEGARDNER
The formation and early differentiation of sea urchin gonads 280
KANESHIRO, EDNA S. AND RICHARD D. KARP
The ultrastructure of coelomocytes of the sea star *Dermasterias imbricata* 295
MARCUS, NANCY H.
Photoperiodic control of diapause in the marine calanoid copepod *Labidocera aestiva* 311
MATSUMOTO, GEN AND JUNICHI SHIMADA
Further improvement upon maintenance of adult squid (*Doryteuthis bleekeri*) in a small circular and closed-system aquarium tank 319
McCORKLE, SUSAN AND THOMAS H. DIETZ
Sodium transport in the freshwater Asiatic clam *Corbicula fluminea* 325
MERCANDO, NEIL A. AND CHARLES F. LYTLE
Specificity in the association between *Hydractinia echinata* and sympatric species of hermit crabs 337
MILLER, CHARLES B., DAVID M. NELSON, ROBERT R. L. GUILLARD, AND BONNIE L. WOODWARD
Effects of media with low silicic acid concentrations on tooth formation in *Acartia tonsa* Dana (Copepoda, Calanoida) 349
MORAN, WILLIAM M. AND RICHARD E. TULLIS
Ion and water balance of the hypo- and hyperosmotically stressed chiton *Mopalia muscosa* 364
OHTSU, KOHZOH
Electrical activities in the subtentacular region of the anthomedusan *Spirocodon saltatrix* (Tilesius) 376
RAHAT, M. AND ORIT ADAR
Effect of symbiotic zooxanthellae and temperature on budding and strobilation in *Cassiopeia andromeda* (Eschscholz) 394
SULKIN, S. D., W. VAN HUEKELEM, P. KELLY, AND L. VAN HUEKELEM
The behavioral basis of larval recruitment in the crab *Callinectes sapidus* Rathbun: A laboratory investigation of ontogenetic changes in geotaxis and barokinesis 402
TURNER, KATHERINE AND TIMOTHY A. LYERLA
Electrophoretic variation in sympatric mud crabs from North Inlet, South Carolina 418
YOUNG, CRAIG M. AND LEE F. BRAITHWAITE
Orientation and current-induced flow in the stalked ascidian *Styela montereyensis* 428
ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY 441

No. 3, DECEMBER 1980

ERRATUM 503

Invited Article:

ALKON, DANIEL L.
Cellular analysis of a gastropod (*Hermissenda crassicornis*) model of associative learning 505

CONTENTS

ALEXANDER, DAVID E., AND JOE GHIOLD
The functional significance of the lunules in the sand dollar, *Mellita quinquiesperforata* 561

BANG, BETSY G., AND FREDERIK B. BANG
The urn cell complex of *Sipunculus nudus:* A model for study of mucus-stimulating substances 571

BANNON, GARY A., AND GEORGE GORDON BROWN
Ultrastructural characteristics of the non-expanded and expanded extra-embryonic shell of the horseshoe crab, *Limulus polyphemus* L. 582

BOUSQUETTE, GEORGE D.
The larval development of *Pinnixa longipes* (Lockington, 1877) (Brachyura: Pinnotheridae), reared in the laboratory 592

BRETOS, MARTA
Age determination in the keyhole limpet *Fissurella crassa* Lamarck (Archaeogastropoda: Fissurellidae), based on shell growth rings 606

CASE, JAMES F.
Courting behavior in a synchronously flashing, aggregative firefly, *Pteroptyx tener* 613

COSTA, CHARLES J., SIDNEY K. PIERCE, AND M. KIM WARREN
The intracellular mechanism of salinity tolerance in polychaetes: Volume regulation by isolated *Glycera dibranchiata* red coelomocytes 626

FISHER, CHARLES R., JR., AND ROBERT K. TRENCH
In vitro carbon fixation by *Prochloron* sp. isolated from *Diplosoma virens* 636

GOODENOUGH, JUDITH E., AND VICTOR G. BRUCE
The effects of caffeine and theophylline on the phototactic rhythm of *Chlamydomonas reinhardii* 649

HAWKINS, CLIFFORD J., PAULINE M. MEREFIELD, DAVID L. PARRY, WILTON R. BIGGS, AND JAMES H. SWINEHART
Comparative study of the blood plasma of the ascidians *Pyura stolonifera* and *Ascidia ceratodes* 656

HAWKINS, CLIFFORD J., DAVID L. PARRY, AND CRAIG PIERCE
Chemistry of the blood of the ascidian *Podoclavella moluccensis* 669

LEE, PAUL H., AND JUDITH S. WEIS
Effects of magnetic fields on regeneration in fiddler crabs 681

MCNAMARA, JOHN C., GLORIA S. MOREIRA, AND PLINIO S. MOREIRA
Respiratory metabolism of *Macrobrachium olfersii* (Wiegmann) zoeae during the moulting cycle from eclosion to first ecdysis 692

MITTENTHAL, JAY EDWARD
On the form and size of crayfish legs regenerated after grafting 700

MITTENTHAL, JAY EDWARD, MARY C. OLSON, AND GLENNA D. CUMMINGS
Morphology of the closer muscles in normal and homoeotic legs of crayfish 714

MORI, TAKAO, TEIZO TSUCHIYA, AND SHONAN AMEMIYA
Annual gonadal variation in sea urchins of the orders Echinothurioida and Echinoida 728

STUNKARD, HORACE W.
The morphology, life history, and systematic relations of *Tubulovesicula pinguis* (Linton, 1940) Manter, 1947 (Trematoda: Hemiuridae) 737

WERMUTH, JEROME F.
Gamma radiation and hydranth longevity in *Campanularia flexuosa:* Age-dependency of dose-response function 752
YUDIN, ASHLEY I., RICHARD A. DIENER, W. H. CLARK, JR., AND ERNEST S. CHANG
Mandibular gland of the blue crab, *Callinectes sapidus* 760
INDEX TO VOLUME 159 773

Volume 159 Number 1

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Marine Biological Laboratory
LIBRARY
SEP 2 2 1980
Woods Hole, Mass.

Editorial Board

DANIEL L. ALKON, National Institutes of Health and Marine Biological Laboratory
FREDERICK B. BANG, Johns Hopkins University
EDWARD M. BERGER, Dartmouth College
STEPHEN C. BROWN, State University of New York at Albany
HARLYN O. HALVORSON, Brandeis University
J. B. JENNINGS, University of Leeds
MEREDITH L. JONES, Smithsonian Institution
GEORGE O. MACKIE, University of Victoria
JOEL L. ROSENBAUM, Yale University
HOWARD A. SCHNEIDERMAN, Monsanto Company
F. JOHN VERNBERG, University of South Carolina
E. O. WILSON, Harvard University

Managing Editor: CHARLES B. METZ, University of Miami

AUGUST, 1980

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, \$10.00. Subscription per volume (three issues), \$27.00, (this is \$54.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and to Dr. Charles B. Metz, Institute For Molecular and Cellular Evolution, University of Miami, 521 Anastasia, Coral Gables, Florida 33134 during the remainder of the year. Assistant editor, Susan Schwartz.

Copyright © 1980, by the Marine Biological Laboratory

Second-class postage paid at Woods Hole, Mass., and additional mailing offices.
ISSN 0006-3185

INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except for a limited number of solicited review papers which may be accepted after formal refereeing), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts should be submitted in triplicate. The original must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, $8\frac{1}{2}$ by 11 inches. They should be carefully proof-read and errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least $1\frac{1}{2}$ inches should be allowed. Manuscripts should be divided into the following components: Title page, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Summary, Literature Cited, Tables (with consecutive Roman numerals) and Figure Legends (with consecutive Arabic numerals).

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed after the Literature Cited. Because of the high cost of setting such material in type authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

4. ***Literature Cited.*** The list of references should be headed LITERATURE CITED, should conform in punctuation and arrangement to the style of recent issues of THE BIOLOGICAL BULLETIN, and must be typed *double-spaced* on separate pages. Note that citations should

Continued on Cover Three

Vol. 159, No. 1 August 1980

THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

EIGHTY-SECOND REPORT, FOR THE YEAR 1979—NINETY-SECOND YEAR

I. TRUSTEES AND STANDING COMMITTEES 1
II. MEMBERS OF THE CORPORATION 5
III. CERTIFICATE OF ORGANIZATION 35
IV. ARTICLES OF AMENDMENT 36
V. BYLAWS 37
VI. REPORT OF THE DIRECTOR 42
VII. REPORT OF THE TREASURER 49
VIII. REPORT OF THE LIBRARIAN 62
IX. EDUCATIONAL PROGRAMS 62
  1. SUMMER 62
  2. JANUARY 70
  3. SHORT COURSES 75
X. RESEARCH AND TRAINING PROGRAMS 80
  1. SUMMER 80
  2. YEAR-ROUND 89
XI. HONORS 94
  1. FRIDAY EVENING LECTURES 94
  2. FELLOWSHIPS AND SCHOLARSHIPS 95
XII. INSTITUTIONS REPRESENTED 95
XIII. LABORATORY SUPPORT STAFF 99

I. TRUSTEES

Including Action of the 1979 Annual Meeting

OFFICERS

PROSSER GIFFORD, *Chairman of the Board of Trustees*, Woodrow Wilson International Center for Scholars, Smithsonian Building, Washington, D. C. 20560

GERARD SWOPE, JR., *Honorary Chairman of the Board of Trustees*, Croton-on-Hudson, New York 10520. (Deceased September 27, 1979)

DENIS M. ROBINSON, *Honorary Chairman of the Board of Trustees*, High Voltage Engineering Corporation, Burlington, Massachusetts 01803

Copyright © 1980, by the Marine Biological Laboratory
Library of Congress Card No. A38-518
(ISSN 0006-3185)

Robert Mainer, *Treasurer*, Boston Company, One Boston Place, Boston, Massachusetts 02106

Paul R. Gross, *President of the Corporation and Director of the Laboratory*, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Gerald S. Fischbach, *Clerk of the Corporation*, Harvard Medical School, Boston, Massachusetts 02215

Emeriti

Philip B. Armstrong, 51 Elliott Place, Rutherford, New Jersey 07070
Eric G. Ball, Marine Biological Laboratory (Deceased September 4, 1979)
Dugald E. S. Brown, 38 Whitman Road, Woods Hole, Massachusetts 02543
Frank A. Brown, Jr., Woods Hole, Massachusett 02543
John B. Buck, National Institutes of Health
Aurin Chase, Princeton University
Anthony C. Clement, Emory University
Kenneth S. Cole, 2404 Loring Street, San Diego, California 92109
Arthur L. Colwin, University of Miami
Laura H. Colwin, University of Miami
D. Eugene Copeland, Marine Biological Laboratory
Sears Crowell, Indiana University
Harry Grundfest, College of Physicians and Surgeons
Teru Hayashi, University of Bonn, West Germany
Hope Hibbard, Oberlin College
Lewis Kleinholz, Reed College
M. E. Krahl, Tucson, Arizona
Douglas Marsland, Cockeysville, Maryland 21030
Harold H. Plough, Amherst, Massachusetts
C. Ladd Prosser, University of Illinois
John S. Rankin, Jr., Ashford, Connecticut
A. C. Redfield, Woods Hole, Massachusetts
S. Meryl Rose, Waquoit, Massachusetts
Mary Sears, Woods Hole, Massachusetts
Carl C. Speidel, University of Virginia
H. Burr Steinbach, Woods Hole, Massachusetts
Albert Szent-Gyorgyi, Marine Biological Laboratory
W. Randolph Taylor, University of Michigan
George Wald, Harvard University

Class of 1983

Nina Allen, Dartmouth College
Hays Clark, Avon Products, Incorporated
Dennis Flanagan, *Scientific American*
William T. Golden, New York, New York
Philip Grant, University of Oregon
Joel Rosenbaum, Yale University
Ann Stuart, University of North Carolina, Chapel Hill
Andrew Szent-Gyorgyi, Brandeis University
Kensal Van Holde, Oregon State University

Class of 1982

Everett Anderson, Harvard Medical School
George H. A. Clowes, Jr., The Cancer Research Institute, Boston
Ellen R. Grass, The Grass Foundation
John P. Kendall, Boston, Massachusetts

Edward A. Kravitz, Harvard Medical School
Hans Laufer, University of Connecticut
Marjorie R. Stetten, National Institutes of Health
Walter S. Vincent, University of Delaware
J. Richard Whittaker, Wistar Institute

Class of 1981

John M. Arnold, University of Hawaii
Jane Fessenden, Marine Biological Laboratory
Helen Homans Gilbert, Dover, Massachusetts
Stephen W. Kuffler, Harvard Medical School
Maurice Lazarus, Federated Department Stores, Boston
George Pappas, University of Illinois Medical Center
W. D. Russell-Hunter, Syracuse University
Raymond E. Stephens, Marine Biological Laboratory

Class of 1980

Philip B.Dunham, Syracuse University
Timothy H. Goldsmith, Yale University
Benjamin Kaminer, Boston University
George Mullen, Watertown, Massachusetts
Keith R. Porter, University of Colorado
Lionel I. Rebhun, University of Virginia
W. Nicholas Thorndike, Boston, Massachusetts
Edward O. Wilson, Harvard University

STANDING COMMITTEES

Executive Committee of the Board of Trustees

Prosser Gifford *ex officio*
Paul R. Gross, *ex officio*
Robert Mainer, *ex officio*
Joel Rosenbaum, 1982
Marjorie R. Stetten, 1982
Edward A. Kravitz, 1981
Kensal Van Holde, 1981
Philip Grant, 1980
Hans Laufer, 1980

Budget Committee

John M. Arnold, *Chairman*
George H. A. Clowes, Jr.
Paul R. Gross, *ex officio*
William T. Golden
Robert Mainer, *ex officio*
Jerome Schiff
Homer P. Smith, *ex officio*
Walter S. Vincent

Buildings and Grounds Committee

Francis Hoskin, *Chairman*
Frank Child
Lawrence B. Cohen
Alan Fein
Daniel Gilbert
Robert Gunning, *ex officio*
Audrey E. V. Haschemeyer
Frank Longo
Robert D. Prusch
Ronald Przybylski
Allen Schuetz
Evelyn Spiegel

Computer Committee

John Hobbie, *Chairman*
William J. Adelman
Francis P. Bowles
Allahverdi Farmanfarmaian
E. F. MacNichol, Jr.
Melvin Rosenfeld, Jr.
Constantine Tollios

Employee Relations Committee

Joan Howard, *Chairman*
Carol Eberhard
Charlotte Frank
Judith Grassle
Lewis Lawday
Donald Lehy
Raymond Stephens

Housing, Food Service, and Day Care Committee

Ann Stuart, *Chairman*
Dan Alkon
Nina Allen
Robert Barlow
Mona Gross
Ronald Joyner
Aimlee Laderman
Brian Salzberg
Homer P. Smith, *ex officio*

Instruction Committee

Benjamin Kaminer, *Chairman*
Daniel Alkon
Robert D. Allen
Esther Goudsmit
Arthur Humes
Robert K. Josephson
Morton Maser, *ex officio*
Merle Mizell
Joel Rosenbaum
Sheldon J. Segal
George Woodwell

Investment Committee

W. Nicholas Thorndike, *Chairman*
Prosser Gifford, *ex officio*
William T. Golden
Maurice Lazarus
John Arnold
Robert Mainer, *ex officio*

Library Committee

Edward A. Adelberg, *Chairman*
Frank M. Child
Bernard Davis
John Dowling
Jane Fessenden, *ex officio*
Nicholas P. Fofonoff
J. W. Hastings
Shinya Inoue
Stephen W. Kuffler
Luigi Mastroianni, Jr.
Robert Morse
Arthur Pardee
Keith R. Porter
Sheldon J. Segal
Derek W. Spencer
Marjorie R. Stetten
Stanley Watson
Carolyn P. Winn, *ex officio*

Macy Scholarship Committee

William W. Sutton, *Chairman*
Lowell Davis
Morton D. Maser, *ex officio*
James Perkins
Edgar E. Smith
James Townsel
Walter S. Vincent
Charles Walker

Marine Resources Committee

Sears Crowell, *Chairman*
Carl J. Berg
June Harrigan
Tom Humphreys
Jack Levin
Cyrus Levinthal
Robert Prendergast
Robert D. Prusch
John S. Rankin
John Valois, *ex officio*
Jonathan Wittenberg

Radiation Committee

Walter S. Vincent, *Chairman*
Eugene Bell
Francis P. Bowles
Richard L. Chappell
Paul DeWeer
John Hobbie
E. F. MacNichol, Jr.
Morton D. Maser, *ex officio*
Harris Ripps

Research Services Committee

Nina S. Allen, *Chairman*
Francis P. Bowles
Samuel S. Koide
E. F. MacNichol, Jr.
Morton D. Maser, *ex officio*
Toshio Narahashi
George Pappas
Jerome Schiff
R. Bruce Szamier
Jay Wells
Seymour Zigman

Research Space Committee

Timothy Goldsmith, *Chairman*
Clay Armstrong
John Arnold
Arthur DuBois
Philip Grant
Frederick Grassle
George Langford
Morton D. Maser, *ex officio*
Joel Rosenbaum
Joan Ruderman
Brian Salzberg
Ann Stuart
George Woodwell

Safety Committee

Robert Gunning, *Chairman ex officio*
Daniel Alkon
Lewis Lawday
Donald Lehy
E. F. MacNichol, Jr.
Morton D. Maser, *ex officio*
Raymond Stephens
Paul Steudler
Frederick Thrasher
Jay Wells

II. MEMBERS OF THE CORPORATION

Including Action of the 1979 Annual Meeting

Life Members

Adolph, Dr. Edward F., University of Rochester, School of Medicine and Dentistry, Rochester, New York 14642

Beams, Dr. Harold W., Department of Zoology, University of Iowa, Iowa City, Iowa 52242

Behre, Dr. Ellinor H., Black Mountain, North Carolina 28711

Bertholf, Dr. Lloyd M., Westminster Village Apt. 2114, 2025 E. Lincoln St., Bloomington, Illinois 61701
Bishop, Dr. David W., Department of Physiology, Medical College of Ohio, Toledo, Ohio 43699
Bold, Dr. Harold C., Department of Botany, University of Texas, Austin, Texas 78712
Bridgman, Dr. A. Josephine, 715 Kirk Rd., Decatur, Georgia 30030
Brown, Dr. Dugald E. S., 38 Whitman Rd., Woods Hole, Massachusetts 02543
Burdick, Dr. C. Lalor, 900 Barley Drive, Barley Mill Court, Wilmington, Delaware 19807
Carpenter, Dr. Russell L., 60 Lake St., Winchester, Massachusetts 01890
Chase, Dr. Aurin, Professor of Biology, Emeritus, Princeton University, Princeton, New Jersey 08540
Clarke, Dr. George L., 44 Juniper Road, Belmont, Massachusetts 02178
Clement, Dr. Anthony C., Department of Biology, Emory University, Atlanta, Georgia 30322
Cole, Dr. Kenneth S., 2404 Loring St., San Diego, California 92109
Colwin, Dr. Arthur L., 320 Woodcrest Rd., Key Biscayne, Florida 33149
Colwin, Dr. Laura H., 320 Woodcrest Rd., Key Biscayne, Florida 33149
Copeland, Dr. D. E., 41 Fern Lane, Woods Hole, Massachusetts 02543
Costello, Dr. Helen M., 507 Monroe St., Chapel Hill, North Carolina 27514
Crouse, Dr. Helen V., Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306
Diller, Dr. Irene C., 2417 Fairhill Avenue, Glenside, Pennsylvania 19038
Diller, Dr. William F., 2417 Fairhill Avenue, Glenside, Pennsylvania 19038
Elliott, Dr. Alfred M., P. O. Box 564, Woods Hole, Massachusetts 02543
Ferguson, Dr. James K. W., 56 Clarkson St., Thornhill, Ontario, L4J 2B4 Canada
Fischer, Dr. Ernst, M.D., 3110 Manor Drive, Richmond, Virginia 23230
Fries, Dr. Erik F. B., 3870 Leafy Way, Miami, Florida 33133
Gray, Dr. Irving E., Department of Zoology, Duke University, Durham, North Carolina 27701
Grundfest, Dr. Harry, Department of Neurology, Columbia University, College of Physicians and Surgeons, New York, New York 10032
Guttman, Dr. Rita, 75 Henry St., Brooklyn, New York 11210
Hamburger, Dr. Viktor, Professor Emeritus, Washington University, St. Louis, Missouri 63130
Hamilton, Dr. Howard L., Department of Biology, University of Virginia, Charlottesville, Virginia 22901
Hartline, Dr. H. Keffer, The Rockefeller University, New York, New York 10021
Hibbard, Dr. Hope, 143 E. College St., Apt. 309, Oberlin, Ohio 44074
Hisaw, Dr. F. L., 5925 S. W. Plymouth Drive, Corvallis, Oregon 97330
Hollaender, Dr. Alexander, Associated University, Inc., 1717 Massachusetts Ave., N. W., Washington, D. C. 20036
Hughes-Schrader, Dr. Sally, Department of Zoology, Duke University, Durham, North Carolina 27706
Irving, Dr. Laurence, University of Alaska, College, Alaska 99701
Johnson, Dr. Frank H., Department of Biology, Princeton University, Princeton, New Jersey 08540
Kaan, Dr. Helen, 62 Locust St., Apt. 244, Falmouth, Massachusetts 02540

Kahler, Robert, P. O. Box 423, Woods Hole, Massachusetts 02543
Kille, Dr. Frank R., 500 Osceola Ave., Winter Park, Florida 32789
Kleinholz, Dr. Lewis, Department of Biology, Reed College, Portland, Oregon 97202
Lochhead, Dr. John H., 49 Woodlawn Rd., London, SW6 6PS, England U. K.
Lynn, Dr. W. Gardner, Department of Biology, Catholic University of America, Washington, D. C. 20017
Magruder, Dr. Samuel R., 270 Cedar Lane, Paducah, Kentucky 42001
Malone, Dr. E. P., 6610 North 11th Street, Philadelphia, Pennsylvania 19126
Manwell, Dr. Reginald D., Department of Biology, Syracuse University, Syracuse, New York 13210
Marsland, Dr. Douglas, Broadmead N12, 13801 York Rd., Cockeysville, Maryland 21030
Miller, Dr. James A., 307 Shorewood Dr., E. Falmouth, Massachusetts 02536
Milne Dr. Lorus J., Department of Zoology, University of New Hampshire, Durham, New Hampshire 03824
Moul, Dr. E. T., 42 F. R. Lillie Rd., Woods Hole, Massachusetts 02543
Nachmansohn, Dr. David, M.D., Department of Neurology, College of Physicians and Surgeons, Columbia University, 630 W. 168th St., New York, New York 10032
Nicoll, Dr. Paul A., 6636 E. Street Rd. 46, Bloomington, Indiana 47401
Page, Dr. Irving H., Box 516, Hyannisport, Massachusetts 02647
Plough, Dr. Harold H., 31 Middle Street, Amherst, Massachusetts 01002
Pollister, Dr. A. W., Box 23, Dixfield, Maine 04224
Pond, Samuel E., P. O. Box 63, E. Winthrop, Maine 04343
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania 19174
Prytz, Dr. Margaret McD., 21 McCouns Lane, Oyster Bay, New York 11771
Renn, Dr. Charles E., Route 2, Hampstead, Maryland 21074
Reznikoff, Dr. Paul, M.D., 11 Brooks Rd., Woods Hole, Massachusetts 02543
Richards, Dr. A. Glenn, Department of Entomology, University of Minnesota, St. Paul, Minnesota 55101
Richards, Dr. Oscar W., Pacific University, Forest Grove, Oregon 97116
Scharrer, Dr. Berta, Department of Anatomy, Albert Einstein College of Medicine, 1300 Morris Pkwy., New York, New York 10461
Schmitt, Dr. F. O., 165 Allen Dale St., Jamaica Plain, Massachusetts 02130
Shemin, Dr. David, Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, Illinois 60201
Sichel, Dr. Elsa K., 4 Whitman Rd., Woods Hole, Massachusetts 02543
Smith, Dr. Dietrich C., 216 Oak Forest Ave., Catonsville, Maryland 21228
Sonneborn, Dr. T. M., Department of Zoology, Indiana University, Bloomington, Indiana 47401
Speidel, Dr. Carl D., 1873 Field Rd., Charlottesville, Virginia 22093
Steinbach, Dr. H. B., One Bell Tower Lane, Woods Hole, Massachusetts 02543
Straus, Dr. W. L., Jr., Department of Anatomy, The Johns Hopkins University Medical School, Baltimore, Maryland 21205
Stunkard, Dr. Horace W., American Museum of Natural History, Central Park West at 79th Street, New York, New York 10024
Taylor, Dr. W. Randolph, Department of Botany, University of Michigan, Ann Arbor, Michigan 48109
TeWinkel, Dr. Lois E., 4 Sanderson Ave., Northampton, Massachusetts 01060

Travis, Dr. Dorothy, 733 Sligo Ave., Apt. 503, Silver Spring, Maryland 20910
Wald, Dr. George, Higgins Professor of Biology, Emeritus, Harvard University, Cambridge, Massachusetts 02138
Wallace, Dr. Louise B., 359 Lytton Ave., Palo Alto, California 94301
Warren, Dr. Herbert S., ℅ Leland C. Warren, 721 Conshohocken State Road, Penn Valley, Pennsylvania 19072
Weiss, Dr. Paul, The Rockefeller University, 66th St. and York Ave., New York, New York 10016
Whiting, Dr. Anna R., Woods Hole, Massachusetts 02543
Wichterman, Dr. Ralph, 31 Buzzards Bay Ave., Woods Hole, Massachusetts 02543
Young, Dr. D. B., Main Street, North Hanover, Massachusetts 02357
Zinn, Dr. Donald J., P. O. Box 589, Falmouth, Massachusetts 02541
Zorzoli, Dr. Anita, Department of Biology, Vassar College, Poughkeepsie, New York 12610
Zweifach, Dr. Benjamin W., ℅ Ames, University of California, La Jolla, California 92037

Regular Members

Abbott, Dr. Marie B., High St., Coventry, Connecticut 06238
Ache, Dr. Barry W., Whitney Marine Laboratory, University of Florida, Rt. 1, Box 121, St. Augustine, Florida 32084
Acheson, Dr. George H., 25 Quisset Ave., Woods Hole, Massachusetts 02543
Adejuwon, Dr. Christopher A., Chemical Pathology Dept., University of Ibadan, Ibadan, Nigeria
Adelberg, Dr. Edward A., Department of Human Genetics, Yale University Medical School, New Haven, Connecticut 06511
Afzelius, Dr. Bjorn, Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden
Alkon, Dr. Daniel, M. D., Head, Section on Neural Systems, Laboratory of Biophysics, Marine Biological Laboratory, Woods Hole, Massachusetts 02543
Allen, Dr. Garland E., Department of Biology, Washington University, St. Louis, Missouri 63130
Allen, Dr. Nina S., Department of Biology, Dartmouth College, Hanover, New Hampshire 03755
Allen, Dr. Robert D., Department of Biology, Dartmouth College, Hanover, New Hampshire 03755
Alscher, Dr. Ruth, Department of Biology, Manhattanville College, Purchase, New York 10577
Amatniek, Ernest, 4797 Boston Post Rd., Pelham Manor, New York 10803
Anderson, Dr. Everett, Department of Anatomy and Laboratories of Human Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02115
Anderson, Dr. J. M., Division of Biological Sciences, Emerson Hall, Cornell University, Ithaca, New York 14853
Armstrong, Dr. Clay M., Department of Physiology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19174
Armstrong, Dr. Peter B., Department of Zoology, University of California, Davis, California 95616

Armstrong, Dr. Phillip B., M.D., 51 Elliott Place, Rutherford, New Jersey 07070

Arnold, Dr. John Miller, Kewalo Marine Lab., Pacific Biomedical Research Center, 41 Ahui St., Honolulu, Hawaii 96813

Arnold, Dr. William A., 102 Balsam Rd., Oak Ridge, Tennessee 37830

Atema, Dr. Jelle, 9 Millfield St., Woods Hole, Massachusetts 02543

Atwood, Dr. Kimball C., 100 Haven Ave., Apt. 21-E, New York, New York 10032

Austin, Dr. Mary L., 506½ North Indiana Avenue, Bloomington, Indiana 47401

Bacon, Robert, P. O. Box 723, Woods Hole, Massachusetts 02543

Baldwin, Dr. Thomas O., Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

Bang, Mrs. Betsy, Department of Pathobiology, The Johns Hopkins University, School of Hygiene, Baltimore, Maryland 21205

Bang, Dr. F. B., Department of Pathobiology, The Johns Hopkins University, School of Hygiene, Baltimore, Maryland 21205

Barker, Dr., Jeffery L., M.D., Bldg. 36, Room 2002, National Institutes of Health, Bethesda, Maryland 20014

Barlow, Dr. Robert B., Jr., Institute for Sensory Research, Syracuse University, Merrill Lane, Syracuse, New York 13210

Bartell, Dr. Clelmer K., 2000 Lake Shore Drive, New Orleans, Louisiana 70122

Barth, Dr. Lucena J., 26 Quisset Ave., Woods Hole, Massachusetts 02543

Bartlett, Dr. James H., Department of Physics, University of Alabama, P. O. Box 1921, University, Alabama 35486

Bauer, Dr. G. Eric, Department of Anatomy, University of Minnesota, Minneapolis, Minnesota 55414

Beauge, Dr. Luis Alberto, Department of Biophysics, University of Maryland School of Medicine, 660 W. Redwood St., Baltimore, Maryland 21201

Beck, Dr. L. V., Department of Pharmacology, Indiana University, School of Experimental Medicine, Bloomington, Indiana 47401

Bell, Dr. Eugene, Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, Massachusetts 02139

Bennett, Dr. Michael V. L., Department of Neuroscience, Albert Einstein College of Medicine, 1300 Morris Park Ave., New York, New York 10461

Bennett, Dr. Miriam F., Department of Biology, Colby College, Waterville, Maine 04901

Berg, Dr. Carl J., Jr., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Berman, Dr. Mones, National Institutes of Health, Theoretical Biology NCI, Bldg. 10, 4B56, Bethesda, Maryland 20014

Bernard, Gary D., Department of Opthalmology and Visual Science, Yale University, 333 Cedar St., New Haven, Connecticut 06510

Berne, Dr. Robert W., University of Virginia School of Medicine, Charlottesville, Virginia 22903

Bernheimer, Dr. Alan W., New York University College of Medicine, New York, New York 10016

Biggers, Dr. John Dennis, Department of Physiology, Harvard Medical School, 25 Shattuck St., Boston, Massachusetts 02115

Bishop, Dr. Stephen H., Department of Zoology, Iowa State University, Ames, Iowa 50010

Blaustein, Mordecai P., Department of Physiology and Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Blum, Dr. Harold F., 404 Locust Lane S., Westchester, Pennsylvania 19380

Bodian, Dr. David, Department of Otolaryngology, The Johns Hopkins University, Traylor Building, Room 424, 1721 Madison St., Baltimore, Maryland 21205

Boettiger, Dr. Edward G., 5 Dunham Pond Rd., Mansfield, Connecticut 06250

Boolootian, Dr. Richard A., President, Science Software System, Inc., 11899 West Pico Blvd., W. Los Angeles, California 90064

Borei, Dr. Hans G., Department of Zoology, University of Pennsylvania, Philadelphia, Pennsylvania 19174

Borgese, Dr. Thomas A., Department of Biology, Lehman College, City University of New York, Bronx, New York 10468

Borisy, Dr. Gary G., Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53715

Bosch, Dr. Herman F., Whipple Hill, Richmond, New Hampshire 03470

Botkin, Dr. Daniel B., Department of Biology, University of California, Santa Barbara, California 93106

Bowen, Dr. Vaughn T., Woods Hole Oceanographic Institution, Redfield Bldg. 3–32, Woods Hole, Massachusetts 02543

Bowles, Dr. Francis P., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Brandt, Dr. Philip Williams, Department of Anatomy, Columbia University, College of Physicians and Surgeons, New York, New York 10032

Brinley, Dr. F. J., Jr., Neurological Disorders Program, NINCDS, 716 Federal Building, Bethesda, Maryland 20205

Brodwick, Dr. Malcolm S., Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

Brooks, Dr. Matilda M., Department of Physiology, University of California, Berkeley, California 94720

Brown, Dr. Frank A., Jr., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Brown, Dr. Jay C., Department of Neurobiology, University of Virginia, Charlottesville, Virginia 22908

Brown, Dr. Joel E., Department of Physiology and Biophysics, Health Sciences Center, State University of New York, Stony Brook, Long Island, New York 11794

Brown, Dr. Stephen C., Department of Biological Sciences, State University of New York, Albany, New York 12222

Buck, Dr. John B., Laboratory of Physical Biology, National Institutes of Health, Bethesda, Maryland 20014

Burbanck, Dr. Madeline Palmer, Box 15134, Atlanta, Georgia 30333

Burbanck, Dr. William D., Box 15134, Atlanta, Georgia 30333

Burdick, Dr. Carolyn J., Department of Biology, Brooklyn College, Brooklyn, New York 11210

Burger, Dr. Max M., Department of Biochemistry, University of Basel, CH. 4056-Klingelbergstrasse 70, Basel, Switzerland

Burky, Dr. Albert J., Department of Biology, University of Dayton, Dayton, Ohio 45469

Burr, Dr. Arthur H., Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

CANDELAS, DR. GRACIELA C., 30 Christopher St., Apt. 5-A, New York, New York 10014

CARLSON, DR. FRANCIS D., Department of Biophysics, The Johns Hopkins University, Baltimore, Maryland 21218

CASE, DR. JAMES F., Department of Biological Sciences, University of California, Santa Barbara, California 93106

CASSIDY, REV. JOSEPH, O.P., Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201

CEBRA, DR. JOHN J., Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

CHAET, DR. ALFRED B., University of West Florida, Pensacola, Florida 32504

CHAMBERS, DR. EDWARD L., Department of Physiology and Biophysics, University of Miami School of Medicine, P. O. Box 52087, Biscayne Annex, Miami, Florida 33152

CHAPPELL, DR. RICHARD L., Department of Biological Sciences, Hunter College, The City University of New York, New York, New York 10021

CHAUNCEY, DR. HOWARD H., 30 Falmouth St., Wellesley Hills, Massachusetts 02181

CHENEY, DR. RALPH H., 45 Coleridge Dr., Falmouth, Massachusetts 02540.

CHILD, DR. FRANK M., Department of Biology, Trinity College, Hartford, Connecticut 06106

CITKOWITZ, DR. ELENA, 410 Livingston St., New Haven, Connecticut 06511

CLARK, DR. A. M., Department of Biological Sciences, University of Delaware, Newark, Delaware 19711

CLARK, DR. ELOISE E., National Science Foundation, 1800 G Street, Washington, D. C. 20550

CLARK, HAYS, Executive Vice-President, Avon Products, Inc., 9 West 57th Street, New York, New York 10019

CLARK, DR. WALLIS H., Aquaculture Program, Rm. 243, Department of Animal Science, University of California, Davis, California 95616

CLAUDE, DR. PHILIPPA, Primate Center, Capital Court, Madison, Wisconsin 53706

CLAYTON, DR. RODERICK K., Section of Genetics, Development and Physiology, Cornell University, Ithaca, New York 14850

CLOWES, DR. GEORGE H. A., JR., The Cancer Research Institute, 194 Pilgrim Rd., Boston, Massachusetts 02215

CLUTTER, DR. MARY, Developmental Biology Program, National Science Foundation, Washington, D. C. 20550

COBB, DR. JEWEL P., Dean, Douglass College, New Brunswick, New Jersey 08903

COHEN, DR. ADOLPH I., Department of Ophthalmology, Washington University, School of Medicine, 660 S. Euclid Ave., St. Louis, Missouri 63110

COHEN, DR. LAWRENCE B., Department of Physiology, Yale University, 333 Cedar St., New Haven, Connecticut 06510

COHEN, DR. SEYMOUR S., Department of Pharmacological Science, State University of New York at Stony Brook, Stony Brook, New York 11790

COHEN, DR. WILLIAM D., Department of Biological Sciences, Hunter College, 695 Park Ave., New York, New York 10021

COLLIER, DR. JACK R., Department of Biology, Brooklyn College, Brooklyn, New York 11210

COOPERSTEIN, DR. SHERWIN J., University of Connecticut, School of Medicine, Farmington Ave., Farmington, Connecticut 06032

Corliss, Dr. John O., Department of Zoology, University of Maryland, College Park, Maryland 20742

Cornell, Dr. Neal W., 6428 Bannockburn Drive, Bethesda, Maryland 20034

Cornman, Dr. Ivor, 10A Orchard Street, Woods Hole, Massachusetts 02543

Couch, Dr. Ernest F., Department of Biology, Texas Christian University, Fort Worth, Texas 76129

Crane, John O., 315 West 106th Street, New York, New York 10025

Cremer-Bartels, Dr. Gertrud, Universitats Augenklinik, 44 Munster, West Germany

Crippa, Dr. Marco, Department de Biologie Animale, Embryologie Moleculaire, 154 route de Malagnou, CH-1224, Chene-Bougeries, Geneve, Switzerland

Crow, Dr. Terry J., Laboratory of Biophysics, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Crowell, Dr. Sears, Department of Zoology, Indiana University, Bloomington, Indiana 47401

Daignault, Alexander T., W. R. Grace Co., 1114 Avenue of the Americas, New York, New York 10036

Dan, Dr. Katsuma, Professor Emeritus, Tokyo Metropolitan University, Meguro-ku, Tokyo, Japan

Danielli, Dr. James F., Life Sciences Department, Worcester Polytechnic Institute, Worcester, Massachusetts 01609

Davis, Dr. Bernard D., M.D., Bacterial Physiology Unit, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115

Daw, Dr. Nigel W., 78 Aberdeen Pl., Clayton, Missouri 63105

DeGroof, Dr. Robert C., Department of Pharmacology, Thomas Jefferson University, 1020 Locust St., Philadelphia, Pennsylvania 19174

DeHaan, Dr. Robert L., Department of Anatomy, Emory University, Atlanta, Georgia 30322

DeLanney, Dr. Louis E., The Jackson Laboratory, Bar Harbor, Maine 04609

DePhillips, Dr. Henry A., Jr., Department of Chemistry, Trinity College, Hartford, Connecticut 06106

Dettbarn, Dr. Wolf-Dietrich, Department of Pharmacology, Vanderbilt University, School of Medicine, Nashville, Tennessee 37217

DeWeer, Dr. Paul J., Department of Physiology, Washington University, School of Medicine, St. Louis, Missouri 63110

Dische, Dr. Zacharias, Columbia University, College of Physicians and Surgeons, 630 W. 165th Street, New York, New York 10032

Dixon, Dr. Keith E., School of Biological Sciences, Flinders University, Bedford Park, South Australia

Dowdall, Dr. Michael J., Department of Biochemistry, University Hospital and Medical School, Clifton Boulevard, Nottingham NG7 2UH, England, U. K.

Dowling, Dr. John E., Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138

Dresden, Dr. Marc H., Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77025

Dudley, Dr. Patricia L., Department of Biological Sciences, Barnard College, Columbia University, New York, New York 10027

Dunham, Dr. Philip B., Department of Biology, Syracuse University, Syracuse, New York 13210

Ebert, Dr. James David, Office of the President, Carnegie Institute of Washington, 1530 P Street N.W., Washington, D. C. 20008

Eckberg, Dr. William R., Department of Zoology, Howard University, Washington, D. C. 20059

Eckert Dr. Roger O., Department of Zoology, University of California, Los Angeles, California 90024

Edds, Dr. Kenneth T., Department of Anatomical Sciences, State University of New York, Buffalo, New York 14214

Edds, Dr. Louise, College of Osteopathic Medicine, Grosvenor Hall, Ohio University, Athens, Ohio 45701

Eder, Dr. Howard A., Albert Einstein College of Medicine, 1300 Morris Park Ave., New York, New York 10461

Edwards, Dr. Charles, Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222

Együd, Dr. Laszlo G., The Sonntag Institute for Cancer Research, Boston College, Chestnut Hill, Massachusetts 02167

Ehrenstein, Dr. Gerald, National Institutes of Health, Bethesda, Maryland 20014

Eichel, Dr. Herbert J., Department of Biological Chemistry, Hahnemann Medical College, Philadelphia, Pennsylvania 19174

Eisen, Dr. Arthur Z., Division of Dermatology, Washington University, School of Medicine, St. Louis, Missouri 63110

Elder, Dr. Hugh Y., Institute of Physiology, University of Glasgow, Glasgow, Scotland, U. K.

Elliott, Dr. Gerald F., The Open University Research Unit, Foxcombe Hall, Berkeley Road, Boar Hill, Oxford, England, U. K.

Epel, Dr. David, Hopkins Marine Station, Pacific Grove, California 93950

Epstein, Dr. Herman T., Department of Biology, Brandeis University, Waltham, Massachusetts 02154

Erulkar, Dr. Solomon D., 318 Kent Rd., Bala Cynwyd, Pennsylvania 19004

Essner, Dr. Edward S., Kresge Eye Institute, Wayne State University, School of Medicine, 540 E. Canfield Ave., Detroit, Michigan 48201

Etienne, Dr. Earl M., Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115

Failla, Dr. Patricia M., Office of the Director, Argonne National Laboratory, Argonne, Illinois 60439

Farmanfarmaian, Dr. Allahverdi, Department of Physiology and Biochemistry, Rutgers University, New Brunswick, New Jersey 08903

Faust, Dr. Robert Gilbert, Department of Physiology, University of North Carolina, Medical School, Chapel Hill, North Carolina 27514

Fein, Dr. Alan, Laboratory of Sensory Physiology, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Ferguson, Dr. F. P., National Institute of General Medical Science, National Institutes of Health, Bethesda, Maryland 20014

Fessenden, Jane, Librarian, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Finkelstein, Dr. Alan, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461

Fischbach, Dr. Gerald, M.D., Department of Pharmacology, Harvard Medical School, 25 Shattuck St., Boston, Massachusetts 02115

Fischman, Dr. Donald A., Department of Anatomy and Cell Biology, State

University of New York, Downstate Medical Center, 450 Clarkson Ave., Brooklyn, New York 11203

Fisher, Dr. J. M., Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Fishman, Dr. Harvey M., Department of Physiology, University of Texas, Medical Branch, Galveston, Texas 77550

Fishman, Dr. Louis, 143 North Grove Street, Valley Stream, New York 11580

Flanagan, Dennis, Editor, *Scientific American*, 415 Madison Ave., New York, New York, 10017

Fox, Dr. Maurice S., Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Fraenkel, Dr. Gottfried S., Department of Entomology, University of Illinois, Urbana, Illinois 61801

Franzini, Dr. Clara, Department of Biology G-5, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19174

Frazier, Dr. Donald T., Department of Physiology and Biophysics, University of Kentucky, School of Medicine, Lexington, Kentucky 40507

Freeman, Dr. Allan R., Professor and Chairman, Department of Physiology, Temple University School of Medicine, 3420 N. Broad St., Philadelphia, Pennsylvania 19140

Freeman, Dr. Gary L., Department of Zoology, University of Texas, Austin, Texas 78710

French, Dr. Robert J., Dept. of Biophysics, University of Maryland, School of Medicine, Baltimore, Maryland 21201

Freygang, Dr. Walter J., Jr., 6247 29th Street, N. W., Washington, D. C. 20015

Fulton, Dr. Chandler M., Department of Biology, Brandeis University, Waltham, Massachusetts 02154

Furshpan, Dr. Edwin J., Department of Neurophysiology, Harvard Medical School, Boston, Massachusetts 02115

Fuseler, Dr. John W., Department of Cell Biology, University of Texas, Medical Branch, 5323 Harry Hines Blvd., Dallas, Texas 75235

Fye, Dr. Paul M., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Gabriel, Dr. Mordecai L., Department of Biology, Brooklyn College, Brooklyn, New York 11210

Gainer, Dr. Harold, Head, Section of Functional Neurochemistry, National Institutes of Health, Bldg. 36, Rm. 2A21, Bethesda, Maryland 20014

Gall, Dr. Joseph G., Department of Biology, Yale University, New Haven, Connecticut 06520

Gelfant, Dr. Seymour, Department of Dermatology, Medical College of Georgia, Augusta, Georgia 30904

Gelperin, Dr. Alan, Department of Biology, Princeton University, Princeton, New Jersey 08540

German, Dr. James L., III, M.D., The New York Blood Center, 310 East 67th Street, New York, New York 10021

Gibbs, Dr. Martin, Institute for Photobiology of Cells and Organelles, Brandeis University, Waltham, Massachusetts 02154

Gibson, Dr. A. Jane, Wing Hall, Cornell University, Ithaca, New York 14850

Gifford, Dr. Prosser, Woodrow Wilson International Center for Scholars, Smithsonian Building, Washington, D. C. 20560

Gilbert, Dr. Daniel L., Laboratory of Biophysics, NINCDS, National Institutes of Health, Building 36, Room 2A29, Bethesda, Maryland 20014

Gilman, Dr. Lauren C., Department of Biology, Box 249118, University of Miami, Coral Gables, Florida 33124

Giudice, Dr. Giovanni, University of Palermo, Via Archirafi 22, Palermo, Italy

Glusman, Dr. Murray, Department of Clinical Psychiatry, Columbia University, 722 W. 168th St., New York, New York 10032

Golden, William T., 40 Wall Street, New York, New York 10005

Goldman, David E., 63 Loop Rd., Falmouth, Massachusetts 02540

Goldman, Dr. Robert D., Department of Biological Sciences, Carnegie-Mellon University, 4400 Fifth Ave., Pittsburgh, Pennsylvania 15213

Goldsmith, Dr. Mary H. M., Department of Biology, Kline Biology Tower, Yale University, New Haven, Connecticut 06520

Goldsmith, Dr. Timothy H., Department of Biology, Yale University, New Haven, Connecticut 06520

Goldstein, Dr. Moise H., Jr., 506 Traylor Bldg., The Johns Hopkins University, School of Medicine, 720 Rutland Ave., Baltimore, Maryland 21205

Gooch, Dr. James L., Department of Biology, Juniata College, Huntington, Pennsylvania 16652

Goodman, Dr. Lesley Jean, Department of Zoology and Comparative Physiology, Queen Mary College, Mile End Rd., London, E1 4 NS, England, U. K.

Gottschall, Dr. Gertrude Y., 315 East 68th Street, Apartment 9M, New York, New York 10021

Goudsmit, Dr. Esther M., Department of Biology, Oakland University, Rochester, Michigan 48063

Gould, Dr. Stephen J., Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts 02138

Graham, Dr. Herbert, 36 Wilson Road, Woods Hole, Massachusetts 02543

Grant, Dr. Phillip, Department of Biology, University of Oregon, Eugene, Oregon 97403

Grass, Albert, The Grass Foundation, 77 Reservoir Road, Quincy, Massachusetts 02170

Grass, Ellen R., The Grass Foundation, 77 Reservoir Road, Quincy, Massachusetts 02170

Grassle, Dr. Judith P., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Green, Dr. James W., Department of Physiology, Rutgers University, New Brunswick, New Jersey 08903

Green, Dr. Jonathan P., Chairman, Department of Biology, Roosevelt University, 430 S. Michigan Ave., Chicago, Illinois 60605

Greenberg, Dr. Michael J., Department of Biological Sciences, Florida State University, Tallahassee, Florida 32306

Gregg, Dr. James H., Department of Zoology, University of Florida, Gainesville, Florida 32601

Greif, Dr. Roger L., Department of Physiology, Cornell University Medical College, New York, New York 10021

Griffin, Dr. Donald R., The Rockefeller University, 1230 York Avenue, New York, New York 10021

Grosch, Dr. Daniel S., Department of Genetics, Gardner Hall, North Carolina State University, Raleigh, North Carolina 27607

Gross, Dr. Paul R., President and Director, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Grossman, Dr. Albert, New York University Medical School, New York, New York 10016

Gunning, A. Robert, 377 Hatchville Road, Hatchville, Massachusetts 02536

Gwilliam, Dr. G. P., Department of Biology, Reed College, Portland, Oregon 97202

Hall, Dr. Zack W., Department of Physiology, University of California, San Francisco, California 94143

Halvorson, Dr. Harlyn O., Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02154

Hamkalo, Dr. Barbara A., Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717

Handler, Dr. Philip, President, National Academy of Sciences, 2101 Constitution Ave. N. W., Washington, D. C. 20418

Hanna, Dr. Robert B., State University of New York, College of Environmental Science and Forestry, Syracuse, New York 13210

Harding, Dr. Clifford V., Jr., Professor and Director of Research, Kresge Eye Institute, Wayne State University, School of Medicine, 540 E. Canfield, Detroit, Michigan 48201

Harosi, Dr. Ferenc I., Laboratory of Sensory Physiology, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Harrigan, Dr. June F., Laboratory of Biophysics, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Harrington, Dr. Glenn W., Department of Microbiology, University of Missouri, School of Dentistry, 650 E. 25th Street, Kansas City, Missouri 64108

Haschemeyer, Dr. Audrey E. V., Department of Biological Sciences, Hunter College, 965 Park Avenue, New York, New York 10021

Hastings, Dr. J. Woodland, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Haxo, Dr. Francis T., Division of Marine Biology, Scripps Institution of Oceanography, University of California, La Jolla, California 92038

Hayashi, Teru, Institute for Cytology, University of Bonn, Ulrich–Haberland Str. 61A, 5300 Bonn, West Germany

Hayes, Dr. Raymond L., Jr., Department of Anatomy, School of Medicine, Morehouse College, 223 Chestnut St. S. W., Atlanta, Georgia 30314

Henley, Dr. Catherine, 5225 Pooks Hill Road, Apt. 1120 North, Bethesda, Maryland 20014

Herndon, Dr. Walter R., 506 Andy Holt Tower, University of Tennessee, Knoxville, Tennessee 37916

Hervey, John P., Box 85, Penzance Road, Woods Hole, Massachusetts 02543

Hessler, Dr. Anita Y., 5795 Waverly Avenue, La Jolla, California 92037

Heuser, Dr. John, M.D., Department of Physiology, School of Medicine, University of California, San Francisco, California 94143

Hiatt, Dr. Howard H., Office of the Dean, Harvard School of Public Health, 677 Huntington Ave., Boston, Massachusetts 02115

Highstein, Dr. Stephen M., Division of Cellular Neurobiology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 14061

Hildebrand, Dr. John G., Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115

Hill, Dr. Robert H., Department of Zoology, University of Rhode Island, Kingston, Rhode Island 02881

Hillman, Dr. Peter, Department of Biology, Hebrew University, Jerusalem, Israel

Hinegardner, Dr. Ralph T., Division of Natural Sciences, University of California, Santa Cruz, California 95060

Hinsch, Dr. Gertrude W., Department of Biology, University of South Florida, Tampa, Florida 33620

Hobbie, Dr. John E., The Ecosystems Center, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Hodge, Dr. Alan J., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Hodge, Dr. Charles, IV, P.O. Box 4095, Philadelphia, Pennsylvania 19118

Hoffman, Dr. Joseph, Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06515

Hoffmann, Dr. Richard J., Department of Zoology, Iowa State University, Ames, Iowa 50011

Hollyfield, Dr. Joe G., Baylor School of Medicine, Texas Medical Center, Houston, Texas 77030

Holtzman, Dr. Eric, Department of Biological Sciences, Columbia University, New York, New York 10027

Holz, Dr. George G., Jr., Department of Microbiology, State University of New York, Upstate Medical Center, Syracuse, New York 13210

Hoskin, Dr. Francis C. G., Department of Biology, Illinois Institute of Technology, Chicago, Illinois 60616

Houghton, Dr. Richard A., III, Ecosystems Center, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Houston, Howard, Preston Ave., Meridan, Connecticut 06450

Hoy, Dr. Ronald R., Section of Neurobiology and Behavior, Cornell University, Ithaca, New York 14850

Hubbard, Dr. Ruth, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Humes, Dr. Arthur G., Boston University Marine Program, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Hummon, Dr. William D., Department of Biology, Ohio University, Athens, Ohio 45701

Humphreys, Dr. Susie Hunt, GRC-NIH, Baltimore City Hospital, Baltimore, Maryland 21224

Humphreys, Dr. Tom D., University of Hawaii, Pacific Biomedical Research Center, 41 Ahui St., Honolulu, Hawaii 96813

Hunter, Dr. Bruce, Provost Office, Tulane University, New Orleans, Louisiana 70118

Hunter, Dr. R. D., Department of Biological Sciences, Oakland University, Rochester, Michigan 48063

Hunziker, H. E., Esq., P.O. Box 547, Falmouth, Massachusetts 02541

Hurwitz, Dr. Charles, Basic Science Research Laboratory, VA Hospital, Albany, New York 12208

Hurwitz, Dr. Jerard, Department of Molecular Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Huxley, Dr. Hugh E., Medical Research Council, Laboratory of Molecular Biology, Cambridge, England, U. K.

Hyde, Dr. Beal B., Department of Botany, University of Vermont, Burlington, Vermont 05401

Ilan, Dr. Joseph, Department of Anatomy, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106

Inoue, Dr. Saduyki, Electron Microscopy Laboratory, McGill University Cancer Center, 3655 Drummond St., Montreal, P. A. Canada HG3 1Y6

Inoué, Dr. Shinya, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Isenberg, Dr. Irving, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

Isselbacher, Dr. Kurt J., Massachusetts General Hospital, Boston, Massachusetts 02714

Issidorides, Dr. Marietta R., Department of Psychiatry, University of Athens, Monis Petraki 8, Athens 140, Greece

Izzard, Dr. Colin S., Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222

Jacobson, Dr. Antone G., Department of Zoology, University of Texas, Austin, Texas 78712

Jaffee, Dr. Lionel, Department of Biology, Purdue University, Lafayette, Indiana 47907

Jahan-Parwar, Behrus, Worcester Foundation for Experimental Biology, 222 Maple Ave., Shrewsbury, Massachusetts 01545

Jannasch, Dr. Holger W., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Jeffery, Dr. William R., Department of Zoology, University of Texas, Austin, Texas 78712

Jenner, Dr. Charles E., Department of Zoology, University of North Carolina, Chapel Hill, North Carolina 27514

Jennings, Dr. Joseph B., Department of Zoology, Baines Wing, University of Leeds, Leeds LS2 9JT, England, U. K.

Jones, Dr. Meredith L., Division of Worms, Museum of Natural History, Smithsonian Institution, Washington, D. C. 20650

Jones, Dr. Raymond F., Department of Biology, State University of New York at Stony Brook, Stony Brook, New York 11753

Josephson, Dr. R. K., School of Biological Sciences, University of California, Irvine, California 92717

Joyner, Dr. Ronald W., Department of Physiology, University of Iowa, Iowa City, Iowa 52242

Kabat, Dr. E. A., Department of Microbiology, Columbia University, College of Physicians and Surgeons, 630 W. 168th St., New York, New York 10032

Kafatos, Dr. Fotis C., The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138

Kaley, Dr. Gabor, Department of Physiology, Basic Sciences Building, New York Medical College, Valhalla, New York 10595

Kaltenbach, Dr. Jane, Department of Biological Sciences, Mount Holyoke College, South Hadley, Massachusetts 01075

Kaminer, Dr. Benjamin, Department of Physiology, Boston University School of Medicine, 80 E. Concord St., Boston, Massachusetts 02118

Kammer, Dr. Ann E., Division of Biology, Kansas State University, Manhattan, Kansas 66502

KANE, DR. ROBERT E., Pacific Biomedical Research Center, 41 Ahui Street, University of Hawaii, Honolulu, Hawaii 96813

KANESHIRO, DR. EDNA S., Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221

KAPLAN, DR. EHUD, The Rockefeller University, 1230 York Ave., New York, New York 10021

KARAKASHIAN, DR. STEPHEN J., 165 West 91st Street, Apt. 16-F, New York, New York 10024

KARUSH, DR. FRED, Department of Microbiology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19174

KATZ, DR. GEORGE M., Department of Neurology, Columbia University, College of Physicians and Surgeons, 630 West 168th Street, New York, New York 10032

KEAN, DR. EDWARD L., Departments of Biochemistry and Ophthalmology, Case Western Reserve University, Cleveland, Ohio 44101

KEMP, DR. NORMAN E., Department of Zoology, University of Michigan, Ann Arbor, Michigan 48104

KENDALL, John P., One Boston Place, Boston, Massachusetts 02108

KEOSIAN, DR. JOHN, P.O. Box 193, Woods Hole, Massachusetts 02543

KETCHUM DR. BOSTWICK H., P.O. Box 32, Woods Hole, Massachusetts 02543

KEYNAN, DR. ALEXANDER, Vice President, Hebrew University, Jerusalem, Israel

KING, DR. THOMAS J., Program Director, Division of Cancer Research, Resources and Center, National Institutes of Health, Bldg. 31, Room 10A03, Bethesda, Maryland 20014

KINGSBURY, DR. JOHN M., Department of Botany, Cornell University, Ithaca, New York 14850

KIRSCHENBAUM, DR. DONALD, Department of Biochemistry, College of Medicine, State University of New York, 450 Clarkson Avenue, Brooklyn, New York 11203

KLEIN, DR. MORTON, Department of Microbiology, Temple University, Philadelphia, Pennsylvania 19122

KLOTZ, DR. I. M., Department of Chemistry, Northwestern University, Evanston, Illinois 60201

KOIDE, DR. SAMUEL S., Population Council, The Rockefeller University, 66th St. and York Ave., New York, New York 10021

KONINGSBERG, DR. IRWIN R., Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, Virginia 22903

KOSOWER, DR. EDWARD M., Department of Chemistry, University of California, La Jolla, California 92093

KRAHL, DR. M. E., 2783 W. Casas Circle, Tucson, Arizona 85704

KRANE, DR. STEPHEN M., Massachusetts General Hospital, Boston, Massachusetts 02114

KRASSNER, DR. STUART MITCHELL, Department of Developmental and Cell Biology, University of California, Irvine, California 92717

KRAUSS, DR. ROBERT, FASEB, 9650 Rockville Pike, Bethesda, Maryland 20014

KRAVITZ, DR. EDWARD A., Department of Neurobiology, Harvard Medical School, 25 Shattuck St., Boston, Massachusetts 02115

KRIEBEL, DR. MAHLON E., Department of Physiology, State University of New York, Upstate Medical Center, 766 Irving Ave., Syracuse, New York 13210

KRIEG, DR. WENDELL J. S., 1236 Hinman, Evanston, Illinois 60602

Krupa, Dr. Paul L., Department of Biology, The City College of New York, 139th Street and Convent Avenue, New York, New York 10031

Kuffler, Dr. Stephen W., Department of Neurophysiology, Harvard Medical School, Boston, Massachusetts 02115

Kusano, Dr. Kiyoshi, Department of Biology, Illinois Institute of Technology, 3300 South Federal Street, Chicago, Illinois 60616

LaMarche, Dr. Paul H., M.D., 593 Eddy St., Providence, Rhode Island 02903

Lancefield, Dr. Rebecca C., The Rockefeller University, 1230 York Ave., New York, New York 10021

Landis, Dennis, M.D., Department of Neurology, Massachusetts General Hospital, Boston, Massachusetts 02114

Landowne, Dr. David, Department of Physiology, University of Miami, Miami, Florida 33124

Langford, Dr. George M., Department of Physiology, University of North Carolina, Medical Sciences Research Wing 206 H, Chapel Hill, North Carolina 27514

Lash, Dr. James W., Department of Anatomy, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19174

Laster, Dr. Leonard, M.D., President, University of Oregon, Health Sciences Center, Portland, Oregon 97201

Laufer, Dr. Hans, Biological Sciences Group U-42, University of Connecticut, Storrs, Connecticut 06268

Lauffer, Dr. Max A., Department of Biophysics, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Lawrence, E. Swift, Pawtucket Institution for Savings, 286 Main St., Pawtucket, Rhode Island 02860

Lazarow, Dr. Jane, 221 Woodlawn Ave., St. Paul, Minnesota 55105

Leadbetter, Dr. Edward R., Biological Sciences Group U-42, University of Connecticut, Storrs, Connecticut 06268

Leak, Dr. Lee Virn, Department of Anatomy, Howard University, College of Medicine, Washington, D. C. 20059

Lecar, Dr. Harold, Laboratory of Biophysics, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20014

Lederberg, Dr. Joshua, President, The Rockefeller University, New York, New York 10021

Lee, Dr. John J., Department of Biology, City College of the City University of New York, Convent Avenue and 138th Street, New York, New York 10031

LeFevre, Dr. Paul G., Department of Physiology, Health Sciences Center, East Campus, State University of New York at Stony Brook, Stony Brook, Long Island, New York 11794

Leighton, Dr. Joseph, M.D., Department of Pathology, Medical College of Pennsylvania, 3300 Henry Ave., Philadelphia, Pennsylvania 19129

Lenher, Dr. Samuel, 50-C Cokesbury Village, Hockessin, Delaware 19707

Lerman, Dr. Sidney, Laboratory for Ophthalmic Research, Emory University, Atlanta, Georgia 30322

Lerner, Dr. Aaron B., Yale Medical School, New Haven, Connecticut 06510

Levin, Dr. Jack, M.D., Hematology Division, The Johns Hopkins Hospital, Baltimore, Maryland 21205

Levine, Dr. Rachmiel, M.D., 2024 Canyon Road, Arcadia, California 91006

Levinthal, Dr. Cyrus, Department of Biological Sciences, 908 Schermerhorn Hall, Columbia University, New York, New York 10027

Levitan, Dr. Herbert, Department of Zoology, University of Maryland, College Park, Maryland 20742

Lewin, Dr. Ralph A., Scripps Institution of Oceanography, La Jolla, California 92093

Ling, Dr. Gilbert, 307 Berkeley Road, Merion, Pennsylvania 19066

Linskens, Dr. H. P., Department of Botany, University of Driehuizerweg 200, Nijmegen, The Netherlands

Lipicky, Dr. Raymond J., M.D., 886 College Parkway No. 304, Rockville, Maryland 20850

Little, Dr. E. P., 216 Highland Street, West Newton, Massachusetts 02158

Liuzzi, Dr. Anthony, Department of Physics, University of Lowell, Lowell, Massachusetts 01854

Llinas, Dr. Rodolfo R., Department of Physiology and Biophysics, New York University Medical Center, 550 First Ave., New York, New York 10016

Loewenstein, Dr. Werner R., Department of Physiology and Biophysics, University of Miami, School of Medicine, P. O. Box 016430, Miami, Florida 33101

Loewus, Dr. Frank A., Department of Agricultural Chemistry, Washington State University, Pullman, Washington 99164

Loftfield, Dr. Robert B., Department of Biochemistry, School of Medicine, University of New Mexico, 900 Stanford N. E., Albuquerque, New Mexico 87106

London, Dr. Irving M., 16-512, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Longo, Dr. Frank J., Department of Anatomy, University of Iowa, Iowa City, Iowa 52442

Lorand, Dr. Laszlo, Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, Illinois 60201

Luria, Dr. Salvador E., Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Lynch, Dr. Clara J., 4800 Filmore Avenue, Alexandria, Virginia 22311

Macagno, Dr. Eduardo R., 1003B Fairchild, Columbia University, New York, New York 10027

MacNichol, Dr. Edward F., Jr., Laboratory of Sensory Physiology, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Mahler, Dr. Henry R., Department of Biochemistry, Indiana University, Bloomington, Indiana 47401

Malkiel, Dr. Saul, Sidney Farber Cancer Center, 35 Binney Street, Boston, Massachusetts 02115

Manalis, Dr. Richard S., Department of Physiology, University of Cincinnati, College of Medicine, Eden and Bethesda Aves., Cincinnati, Ohio 45267

Mangum, Dr. Charlotte P., Department of Biology, College of William and Mary, Williamsburg, Virginia 23185

Marks, Dr. Paul A., Health Sciences Center, Room 1802, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, New York 10032

Marsh, Dr. Julian B., Department of Biochemistry and Physiology, Medical College of Pennsylvania, 3300 Henry Ave., Philadelphia, Pennsylvania 19129

Martin, Lowell V., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Maruo, Dr. Takeshi, M.D., Dept. of Obstetrics and Gynecology, Kobe University School of Medicine, Ikuta–ku, Kobe 650, Japan

Maser, Dr. Morton, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Mastroianni, Dr. Luigi, Jr., M.D., Chairman, Department of Obstetrics and Gynecology, Hospital of the University of Pennsylvania, 3400 Spruce St., Philadelphia, Pennsylvania 19174

Mathews, Dr. Rita W., Hunter College, Box 1075, 695 Park Ave., New York, New York 10021

Mautner, Dr. Henry G., Department of Biochemistry and Pharmacology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111

Mauzerall, Dr. David, The Rockefeller University, 66th Street and York Avenue, New York, New York 10021

Maxwell, Dr. Arthur, Provost, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Mazia, Dr. Daniel, Department of Zoology, University of California, Berkeley, California 94720

McCann, Dr. Frances, Department of Physiology, Dartmouth Medical School, Hanover, New Hampshire 03755

McCloskey, Dr. Lawrence R., Department of Biology, Walla Walla College, College Place, Washington 99324

McLaughlin, Jane A., P. O. Box 187, Woods Hole, Massachusetts 02543

McMahon, Dr. Robert F., Department of Biology, University of Texas, Arlington, Texas 76019

McReynolds, Dr. John S., Department of Physiology, University of Michigan, Ann Arbor, Michigan 48104

Meinkoth, Dr. Norman A., Department of Biology, Swarthmore College, Swarthmore, Pennsylvania 19081

Meiss, Dr. Dennis E., Dept. of Biology, Clark University, Worcester, Massachusetts 01610

Melillo, Dr. Jerry M., Ecosystems Center, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Mellon, Dr. DeForest, Jr., Department of Biology, University of Virginia, Charlottesville, Virginia 22903

Metz, Dr. C. B., Institute of Molecular Evolution, University of Miami, 521 Anastasia St., Coral Gables, Florida 33134

Middlebrook, Dr. Robert, 86 Station Road, Burley-In-Warfedale, West Yorks, England, U. K.

Milkman, Dr. Roger D., Department of Zoology, University of Iowa, Iowa City, Iowa 52242

Mills, Dr. Eric Leonard, Institute of Oceanography, Dalhousie University, Halifax, Nova Scotia, Canada

Mills, Robert, 56 Worcester Ct., Falmouth, Massachusetts 02540

Mitchell, Dr. Ralph, Pierce Hall, Harvard University, Cambridge, Massachusetts 02138

Mizell, Dr. Merle, Department of Biology, Tulane University, New Orleans, Louisiana 70118

Monroy, Dr. Alberto, Stazione Zoologica, Villa Communale, Napoli, Italy

Montroll, Dr. Eliott W., Institute for Fundamental Studies, Department of Physics, University of Rochester, Rochester, New York 14627

Moore, Dr. John A., Department of Biology, University of California, Riverside, California 92521

Moore, Dr. John W., Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710

Moore, Dr. Lee E., Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

Moran, Dr. Joseph F., Jr., 23 Foxwood Drive, RR# 1, Eastham, Massachusetts 02642

Morin, Dr. James G., Department of Biology, University of California, Los Angeles, California 90024

Morrell, Dr. Frank, Department of Neurological Sciences, Rush Medical Center, 1753 W. Congress Pkwy., Chicago, Illinois 60612

Morrill, Dr. John B., Jr., Division of Natural Sciences, New College, Sarasota, Florida 33580

Morse, Dr. Richard Stetson, 193 Winding River Road, Wellesley, Massachusetts 02181

Morse, Robert W., Associate Director, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Moscona, Dr. A. A., Department of Biology, University of Chicago, Chicago, Illinois 60627

Mote, Dr. Michael I., Department of Biology, Temple University, Philadelphia, Pennsylvania 19122

Mountain, Dr. Isabel M., Vinson Hall #112, 6251 Old Dominion Drive, McLean, Virginia 22101

Mullen, George, Belknap and McLain, 650 Pleasant St., Watertown, Massachusetts 02172

Musacchia, Dr. Xavier J., Graduate School, University of Louisville, Louisville, Kentucky 40208

Nabrit, Dr. S. M., 686 Beckwith Street S. W., Atlanta, Georgia 30314

Nace, Dr. Paul Foley, 5 Bowditch Rd., Woods Hole, Massachusetts 02543

Nakajima, Dr. Shigehiro, Department of Biological Sciences, Purdue University, W. Lafayette, Indiana 47907

Nakajima, Dr. Yasuko, Department of Biological Sciences, Purdue University, W. Lafayette, Indiana 47907

Narahashi, Dr. Toshio, Department of Pharmacology, Medical Center, Northwestern University, 303 E. Chicago Ave., Chicago, Illinois 60611

Nasatir, Dr. Maimon, Department of Biology, University of Toledo, Toledo, Ohio 43606

Nelson, Dr. Leonard, Department of Physiology, Medical College of Ohio at Toledo, Toledo, Ohio 43699

Nelson, Dr. Margaret C., Section on Neurobiology and Behavior, Cornell University, Ithaca, New York 14850

Nicholls, Dr. John G., Department of Neurobiology, Stanford University, Stanford, California 94305

Nicosia, Dr. Santo V., M.D., Department of Obstetrics and Gynecology, Division of Reproductive Biology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174

Nielsen, Dr. Jennifer B. K., Waksman Institute for Microbiology, Rutgers University, Piscataway, New Jersey 08854

Noe, Dr. Bryan D., Department of Anatomy, Emory University, Atlanta, Georgia 30345

Ochoa, Dr. Severo, 530 East 72nd Street, New York, New York 10021

Odum, Dr. Eugene, Department of Zoology, University of Georgia, Athens, Georgia 30601

Oertel, Dr. Donata, Department of Neurophysiology, University of Wisconsin, 283 Medical Science Building, Madison, Wisconsin 53706

O'Herron, Jonathan, Lazard Freres and Company, 1 Rockefeller Plaza, New York, New York 10020

Olson, Dr. John M., Department of Biology, Brookhaven National Laboratory, Upton, New York 11973

Oschman, Dr. James L., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Oxford, Dr. Gerry S., Department of Physiology, University of North Carolina, Chapel Hill, North Carolina 27514

Palmer, Dr. John D., Department of Zoology, University of Massachusetts, Amherst, Massachusetts 01002

Palti, Dr. Yoram, Head, Department of Biophysics, University of Maryland, Baltimore, Maryland 21201

Pappas, Dr. George D., Department of Anatomy, College of Medicine, University of Illinois, 808 S. Wood St., Chicago, Illinois 60612

Pardee, Dr. Arthur B., Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Pardy, Dr. Roosevelt L., School of Life Sciences, University of Nebraska, Lincoln, Nebraska 68588

Parmentier, Dr. James L., Department of Anesthesiology, Duke University Medical Center, Durham, North Carolina 27710

Passano, Dr. Leonard M., Department of Zoology, Birge Hall, University of Wisconsin, Madison, Wisconsin 53706

Pearlman, Dr. Alan L., Department of Physiology, School of Medicine, Washington University, St. Louis, Missouri 63110

Pederson, Dr. Thoru, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

Perkins, Dr. C. D., National Academy of Engineering, 2101 Constitution Ave., N. W., Washington, D. C. 20418

Person, Dr. Philip, Special Dental Research Program, Veteran's Administration Hospital, Brooklyn, New York 11219

Peterson, Dr. Bruce J., Ecosystems Center, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Pettibone, Dr. Marian H., Division of Worms, W-213, Smithsonian Institution, Washington, D. C. 20560

Pfohl, Dr. Ronald J., Department of Zoology, Miami University, Oxford, Ohio 45056

Philpott, Dr. Delbert E., Ames Research Center, Moffett Field, California 94035

Pierce, Dr. Sidney K., Jr., Department of Zoology, University of Maryland, College Park, Maryland 20740

Pollard, Dr. Harvey B., National Institutes of Health, F. Bldg. 10, Rm. 10B17, Bethesda, Maryland 20014

Pollard, Dr. Thomas D., M.D., Director, Department of Cell Biology and

Anatomy, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, Maryland 21205

Pollock, Dr. Leland W., Department of Zoology, Drew University, Madison, New Jersey 07940

Porter, Dr. Beverly H., 14433 Taos Court, Wheaton, Maryland 20906

Porter, Dr. Keith R., 748 11th Street, Boulder, Colorado 80302

Potter, Dr. David, Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115

Potter, Dr. H. David, Neural Sciences Program, Chemistry Building, Indiana University, Bloomington, Indiana 47401

Potts, Dr. William T., Department of Biology, University of Lancaster, Lancaster, England, U. K.

Poussart, Dr. Denis, Department of Electrical Engineering, Université Laval, Quebec, Canada

Prendergast, Dr. Robert A., Department of Pathology and Ophthalmology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Price, Dr. Carl A., Waksman Institute of Microbiology, Rutgers University, P. O. Box 759, Piscataway, New Jersey 08854

Price, Dr. Christopher H., Biological Science Center, 2 Cummington Street, Boston, Massachusetts 02215

Prior, Dr. David James, Department of Biological Sciences, University of Kentucky, Lexington, Kentucky 40506

Prosser, Dr. C. Ladd, Department of Physiology and Biophysics, Burrill Hall 524, University of Illinois, Urbana, Illinois 61801

Provasoli, Dr. Luigi, Haskins Laboratories, 165 Prospect Street, New Haven, Connecticut 06520

Prusch, Dr. Robert D., Division of Biomedical Sciences, Brown University, Providence, Rhode Island 02908

Przybylski, Dr. Ronald J., Department of Anatomy, Case Western Reserve University, Cleveland, Ohio 44101

Rabin, Dr. Harvey, P. O. Box 239, Braddock Hts., Maryland 21714

Ramon, Dr. Fidel, Department of Physiology, Duke University Medical Center, Durham, North Carolina 27706

Rankin, Dr. John S., Box 97, Ashford, Connecticut 06278

Ranzi, Dr. Silvio, Department of Zoology, University of Milan, Via Celonia 10, Milan, Italy

Ratner, Dr. Sarah, Department of Biochemistry, The Public Health Research Institute, 455 First Avenue, New York, New York 10016

Rebhun, Dr. Lionel I., Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, Virginia 22901

Reddan, Dr. John R., Department of Biological Sciences, Oakland University, Rochester, Michigan 48063

Redfield, Dr. Alfred C., 10 Maury Lane, Woods Hole, Massachusetts 02543

Reese, Dr. Thomas S., Head, Section on Functional Neuroanatomy, National Institutes of Health, Bethesda, Maryland 20014

Reiner, Dr. John M., Department of Biochemistry, Albany Medical College of Union University, Albany, New York 12208

Reinisch, Dr. Carol L., Department of Tumor Immunology, Sidney Farber Cancer Institute, 44 Binney St., Boston, Massachusetts 02511

Reuben, Dr. John P., Department of Neurology, Columbia University, College of Physicians and Surgeons, New York, New York 10032

Reynolds, Dr. George Thomas, Department of Physics, Princeton University, Princeton, New Jersey 08540

Rice, Dr. Robert Vernon, Carnegie-Mellon Institute, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213

Rickles, Dr. Frederick R., M.D., University of Connecticut, School of Medicine, VA Hospital, Newington, Connecticut 06111

Ripps, Dr. Harris, Department of Opthalmology, New York University, School of Medicine, 550 First Ave., New York, New York 10016

Roberts, Dr. John L., Department of Zoology, University of Massachusetts, Amherst, Massachusetts 01002

Robinson, Dr. Denis M., 19 Orlando Avenue, Arlington, Massachusetts 02174

Rockstein, Dr. Morris, Department of Physiology, University of Miami School of Medicine, P. O. Box 016430, Miami, Florida 33101

Ronkin, Dr. Raphael E., 3212 McKinley St., N. W., Washington, D. C. 20015

Rose, Dr. Birgit, Department of Physiology, University of Miami School of Medicine, P. O. Box 016430, Miami, Florida 33101

Rose, Dr. S. Meryl, Box 309W, Waquoit, Massachusetts 02536

Rosenbaum, Dr. Joel L., Kline Biology Tower, Yale University, New Haven, Connecticut 06510

Rosenberg, Dr. Evelyn K., Jersey City State College, Jersey City, New Jersey 07305

Rosenberg, Dr. Phillip, Division of Pharmacology, University of Connecticut, School of Pharmacy, Storrs, Connecticut 06268

Rosenbluth, Dr. Jack, Department of Physiology, New York University, School of Medicine, 550 First Avenue, New York, New York 10016

Rosenbluth, Raja, 3380 West 5th Avenue, Vancouver, British Columbia, Canada V6R 1R7

Rosenkranz, Dr. Herbert S., Department of Microbiology, New York Medical College, Valhalla, New York 10595

Roslansky, Dr. John, Box 208, Woods Hole, Massachusetts 02543

Roslansky, Dr. Priscilla F., Box 208, Woods Hole, Massachusetts 02543

Ross, Dr. William N., Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115

Roth, Dr. Jay S., Division of Biological Sciences, Section of Biochemistry and Biophysics, University of Connecticut, Storrs, Connecticut 06268

Rowe, Dorothy, 88 Chestnut St., Boston, Massachusetts 02165

Rowland, Dr. Lewis P., Department of Neurology, Columbia University, College of Physicians and Surgeons, 630 W. 168th St., New York, New York 10032

Rubinow, Dr. Sol I., Department of Biomathematics, Cornell University, Medical College, New York, New York 10012

Ruderman, Dr. Joan V., Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115

Rushforth, Dr. Norman B., Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106

Russell, Dr. John M., Department of Biophysics, University of Texas, Medical Branch, Galveston, Texas 77550

Russell-Hunter, Dr. W. D., Department of Biology, Lyman Hall, Syracuse University, Syracuse, New York 13210

Rustad, Dr. Ronald C., Department of Radiology, Case Western Reserve University, Cleveland, Ohio 44106

Sager, Dr. Ruth, Sidney Farber Cancer Center, 44 Binney St., Boston, Massachusetts 02115

Salmon, Dr. Edward D., Department of Zoology, University of North Carolina, Chapel Hill, North Carolina 27514

Salzberg, Dr. Brian M., Department of Physiology, University of Pennsylvania, 4010 Locust St., Philadelphia, Pennsylvania 19174

Sanders, Dr. Howard L., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Sato, Dr. Hidemi, Sugashima Marine Biological Laboratory, Nagoya University, Sugashima-cho, Toba-shi, Mie-ken, 517, Japan

Saunders, Dr. John W., Jr., Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222

Saz, Dr. Arthur Kenneth, Department of Microbiology, Georgetown University Medical and Dental Schools, 3900 Reservoir Road, N. W., Washington, D. C. 20051

Schachman, Dr. Howard K., Department of Molecular Biology, University of California, Berkeley, California 94720

Schiff, Dr. Jerome A., Institute for Photobiology of Cells and Organelles, Brandeis University, Waltham, Massachusetts 02154

Schlesinger, Dr. R. Walter, Department of Microbiology, Rutgers Medical School, P. O. Box 101, Piscataway, New Jersey 08854

Schmeer, Sister Arline C., American Cancer Research Center and Hospital, 6401 W. Colfax Ave., Denver, Colorado 80214

Schneiderman, Dr. Howard A., Monsanto Co., 800 N. Lindberg Blvd. (D1W), St. Louis, Missouri 63166

Scholander, Dr. P. F., Scripps Institution of Oceanography, La Jolla, California 92038

Schopf, Dr. Thomas J. M., Department of the Geophysical Sciences, University of Chicago, 5734 S. Ellis Avenue, Chicago, Illinois 60637

Schotté, Dr. Oscar E., Department of Biology, Amherst College, Amherst, Massachusetts 01002

Shoukimas, Dr. Jonathan J., Laboratory of Biophysics, NINCDS, Marine Biological Laboratory, Woods Hole, Massachusetts, 02543

Schuel, Dr. Herbert, Department of Anatomical Sciences, State University of New York, Buffalo, New York 14214

Schuetz, Dr. Allen Walter, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21205

Schwartz, Dr. Tobias L., Biological Sciences Group, University of Connecticut, Storrs, Connecticut 06268

Scott, Dr. Allan C., 1 Nudd St., Waterville, Maine 04901

Scott, Dr. George T., Department of Biology, Oberlin College, Oberlin, Ohio 44074

Sears, Dr. Mary, Box 152, Woods Hole, Massachusetts 02543

Segal, Dr. Sheldon J., Director, Population Division, The Rockefeller Foundation, 1133 Avenue of the Americas, New York, New York 10036

Seliger, Dr. Howard H., McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

Selman, Dr. Kelly, Department of Anatomy, College of Medicine, University of Florida, Gainesville, Florida 32601

Senft, Dr. Joseph P., Organic Gardening and Farming Research Center, Box 323, R.D.1, Kutztown, Pennsylvania 19530

Shanklin, Dr. Douglas R., P. O. Box 1267, Gainesville, Florida 32602

Shapiro, Dr. Herbert, 6025 North 13th Street, Philadelphia, Pennsylvania 19141

Shaver, Dr. John R., Department of Zoology, Michigan State University, East Lansing, Michigan 48823

Shepard, Dr. David C., P. O. Box 44, Woods Hole, Massachusetts 02543

Shepro, Dr. David, Department of Biology, Boston University, 2 Cummington Street, Boston, Massachusetts 02215

Sherman, Dr. I. W., Division of Life Sciences, University of California, Riverside, California 92502

Shilo, Dr. Moshe, Head, Department of Microbiological Chemistry, Hebrew University, Jerusalem, Israel

Siegel, Dr. Irwin M., Department of Ophthalmology, New York University Medical Center, 550 First Avenue, New York, New York 10016

Siegelman, Dr. Harold W., Department of Biology, Brookhaven National Laboratory, Upton, New York 11973

Simon, Dr. Eric J., New York University Medical School, 550 First Avenue, New York, New York 10016

Sjodin, Dr. Raymond A., Department of Biophysics, University of Maryland School of Medicine, Baltimore, Maryland 21201

Skinner, Dr. Dorothy M., Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

Slobodkin, Dr. Lawrence B., Department of Biology, State University of New York at Stony Brook, Stony Brook, New York 11790

Smith, Homer P., General Manager, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Smith, Dr. Michael A., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Smith, Paul Ferris, P. O. Box 264, Woods Hole, Massachusetts 02543

Smith, Dr. Ralph I., Department of Zoology, University of California, Berkeley, California 94720

Sonnenblick, Dr. B. P., Department of Zoology and Physiology, Rutgers University, 195 University Avenue, Newark, New Jersey 07102

Sorenson, Dr. Albert L., Department of Physiology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461

Sorenson, Dr. Martha M., Department of Neurology, Columbia University, College of Physicians and Surgeons, New York, New York 10032

Speck, Dr. William T., M.D., Department of Pediatrics, Case Western Reserve University, Cleveland, Ohio 44106

Spector, Dr. A., Black Bldg., Rm. 1516, Columbia University, College of Physicians and Surgeons, New York, New York 10032

Spiegel, Dr. Evelyn, Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755

Spiegel, Dr. Melvin, Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755

Spirtes, Dr. Morris Albert, M.D., Veteran's Administration Hospital, 1601 Perdido Street, New Orleans, Louisiana 70112

Spray, Dr. David C., Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461

STARZAK, DR. MICHAEL E., Department of Chemistry, State University of New York, Binghamton, New York 13901

STEELE, DR. JOHN HYSLOP, Director, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

STEINBERG, DR. MALCOLM, Department of Biology, Princeton University, Princeton, New Jersey 08540

STEINACHER, DR. ANTOINETTE, Department of Biophysics, The Rockefeller University, New York, New York 10021

STEINHARDT, DR. JACINTO, 306 Reiss Bldg., Georgetown University, Washington, D. C. 20007

STEPHENS, DR. GROVER C., School of Biological Sciences, University of California, Irvine, California 92717

STEPHENS, DR. RAYMOND E., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

STETTEN, DR. MARJORIE R., National Institutes of Health, Bldg. 10, 9B-02, Bethesda, Maryland 20014

STETTEN, DR. DEWITT, JR., M.D., PH.D., Senior Scientific Advisor, NIH, Building 16, Room 118, Bethesda, Maryland 20014

STOKES, DR. DARRELL R., Department of Biology, Emory University, Atlanta, Georgia 30322

STRACHER, DR. ALFRED, State University of New York, Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, New York 11203

STREHLER, DR. BERNARD L., 2310 N. Laguna Circle Dr., Agoura, California 91301

STRETTON, DR. ANTHONY O. W., Department of Zoology, University of Wisconsin, Madison, Wisconsin 53706

STUART, DR. ANN E., Medical Sciences Research Wing 206H, Department of Physiology, University of North Carolina, Chapel Hill, North Carolina 27514

SUMMERS, DR. WILLIAM C., Huxley College, Western Washington State College, Bellingham, Washington 98225

SUSSMAN, DR. MAURICE, Department of Life Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

SZABÓ, DR. GEORGE, Harvard School of Dental Medicine, 188 Longwood Avenue, Boston, Massachusetts 02115

SZAMIER, DR. ROBERT BRUCE, Harvard Medical School, Berman-Gund Laboratory, Eye and Ear Infirmary, 243 Charles Street, Boston, Massachusetts 02114

SZENT-GYÖRGYI, DR. ALBERT, Institute for Muscle Research, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

SZENT-GYÖRGYI, DR. ANDREW G., Department of Biology, Brandeis University, Waltham, Massachusetts 02154

TAKASHIMA, DR. SHIRO, Department of Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania 19174

TANZER, DR. MARVIN L., Department of Biochemistry, Box G, University of Connecticut, School of Medicine, Farmington, Connecticut 06032

TASAKI, DR. ICHIJI, Laboratory of Neurobiology, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland 20014

TAYLOR, DR. DOUGLAS L., The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

TAYLOR, DR. ROBERT E., Laboratory of Biophysics, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20014

Taylor, Dr. W. Rowland, 1540 Northbourne Rd., Baltimore, Maryland 21239

Telfer, Dr. William H., Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19174

deTerra, Dr. Noel, Department of Anatomy, Hahnemann Medical College, 230 N. Broad St., Philadelphia, Pennsylvania 19102

Thorndike, W. Nicholas, Wellington Management Company, 28 State St., Boston, Massachusetts 02109

Tiffney, Dr. Wesley N., 226 Edge Hill Rd., Sharon, Massachusetts 02067

Trager, Dr. William, The Rockefeller University, 66th Street and York Avenue, New York, New York 10021

Travis, Dr. D. M., Department of Pharmacology, University of Florida, Gainesville, Florida 32610

Trinkaus, Dr. J. Philip, Osborn Zoological Laboratories, Department of Zoology, Yale University, New Haven, Connecticut 06510

Troll, Dr. Walter, Department of Environmental Medicine, New York University, College of Medicine, New York, New York 10016

Troxler, Dr. Robert F., Department of Biochemistry, Boston University School of Medicine, 80 E. Concord St., Boston, Massachusetts 02118

Turner, Dr. Ruth D., Mollusk Department, Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts 02138

Tweedell, Dr. Kenyon S., Department of Biology, University of Notre Dame, Notre Dame, Indiana 46656

Uretz, Dr. Robert B., Division of Biological Sciences, University of Chicago, 950 E. 59th St., Box 417, Chicago, Illinois 60637

Valiela, Dr. Ivan, Boston University Marine Program, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Valois, John, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Van Holde, Dr. Kensal Edward, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

Villee, Dr. Claude A., Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

Vincent, Dr. Walter S., Chairman, Department of Biological Sciences, University of Delaware, Newark, Delaware 19711

Wainio, Dr. W. W., Box 1059, Nelson Labs., Rutgers Biochemistry, Piscataway, New Jersey 08854

Waksman, Dr. Byron, Department of Pathology, Yale University, New Haven, Connecticut 06510

Walker, Dr. Charles A., 3113 Shamrock South, Tallahasee, Florida 32303

Wall, Dr. Betty J., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Wallace, Dr. Robin A., P. O. Box Y, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

Wang, Dr. A., Bedford Road, Lincoln, Massachusetts 01773

Warner, Dr. Robert C., Department of Molecular and Cell Biology, University of California, Irvine, California 92717

Warren, Dr. Leonard, Department of Therapeutic Research, Anatomy-Chemistry Bldg., Rm. 337, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19174

Waterman, Dr. T. H., 610 Kline Biology Tower, Yale University, New Haven, Connecticut 06520

WATSON, DR. STANLEY WAYNE, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

WEBB, DR. H. MARGUERITE, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

WEBER, DR. ANNEMARIE, M.D., Department of Biochemistry, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19174

WEBSTER, DR. FERRIS, 800 25th Street, N. W., Washington, D. C. 20037

WEIDNER, DR. EARL, Department of Zoology and Physiology, Louisiana State University, Baton Rouge, Louisiana 70803

WEISS, DR. LEON P., Department of Animal Biology, University of Pennsylvania, School of Veterinary Medicine, Philadelphia, Pennsylvania 19174

WEISSMANN, DR. GERALD, School of Medicine, New York University, 550 First Avenue, New York, New York 10016

WERMAN, DR. ROBERT, Neurobiology Unit, Hebrew University, Jerusalem, Israel

WHITTAKER, DR. J. RICHARD, Wistar Institute for Anatomy and Biology, 36th Street at Spruce, Philadelphia, Pennsylvania 19174

WIERCINSKI, DR. FLOYD J., Department of Biology, Northeastern Illinois University, 5500 North St. Louis Ave., Chicago, Illinois 60625

WIGLEY, DR. ROLAND L., National Marine Fisheries Service, Woods Hole, Massachusetts 02543

WILBER, DR. C. G., Chairman, Department of Zoology, Colorado State University, Fort Collins, Colorado 80524

WILSON, DR. DARCY B., Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19174

WILSON, DR. EDWARD O., Department of Zoology, Harvard University, Cambridge, Massachusetts 02138

WILSON, DR. T. HASTINGS, Department of Physiology, Harvard Medical School, Boston, Massachusetts 02115

WILSON, DR. WALTER L., Department of Biology, Oakland University, Rochester, Michigan 48063

WITKOVSKY, DR. PAUL, Department of Ophthalmology, New York University Medical Center, 550 First Ave., New York, New York 10016

WITTENBERG, DR. JONATHAN B., Department of Physiology and Biochemistry, Albert Einstein College of Medicine, New York, New York 10461

WOELKERLING, DR. WILLIAM J., Department of Botany, Latrobe University, Bundoora, Victoria, Australia 3083

WOLF, DR. DON P., University of Pennsylvania, School of Medicine, 314 Medical Labs 6/3, Philadelphia, Pennsylvania 19174

WOODWELL, DR. GEORGE M., Director, The Ecosystems Center, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

WYTTENBACH, DR. CHARLES R., Department of Physiology and Cell Biology, University of Kansas, Lawrence, Kansas 66045

YEH, JAY Z., Department of Pharmacology, Northwestern University Medical School, 303 E. Chicago Ave., Chicago, Illinois 60611

YNTEMA, DR. C. L., Department of Anatomy, State University of New York, Upstate Medical Center, Syracuse, New York 13210

YOUNG, DR. DAVID K., Department of the Navy, NORDA, Code 334, NSTL Station, Mississippi 39529

YPHANTIS, DR. DAVID A., Department of Biochemistry and Biophysics, University of Connecticut, Storrs, Connecticut 06268

Zigman, Dr. Seymour, University of Rochester School of Medicine and Dentistry, 260 Crittenden Boulevard, Rochester, New York 14620

Zimmerman, Dr. A. M., Department of Zoology, University of Toronto, Toronto, Ontario, Canada

Associate Members

Acheson, Dr. and Mrs. George
Ackroyd, Dr. and Mrs. Frederick W.
Adelberg, Dr. and Mrs. Edward A.
Adelman, Dr. and Mrs. William J.
Ahearn, Mr. and Mrs. David C.
Allen, Miss Camilla K.
Allen, Mrs. Robert D.
Amberson, Mrs. William R.
Armstrong, Dr. Philip
Armstrong, Dr. and Mrs. Samuel C.
Arnold, Dr. and Mrs. John
Atwood, Dr. and Mrs. Kimball C.
Bacon, Mr. Robert
Ball, Mrs. Eric G.
Ballantine, Dr. and Mrs. H. T., Jr.
Bang, Dr. and Mrs. Frederik B.
Banks, Mrs. and Mrs. W. L.
Barrows, Mrs. Albert W.
Bennett, Dr. and Mrs. Michael V. L.
Bernstein, Mr. and Mrs. Herman
Bernheimer, Dr. Alan W.
Bigelow, Mrs. Robert
Blackburn, Dr. and Mrs. George L.
Bodeen, Mr. and Mrs. George H.
Boettiger, Dr. and Mrs. Edward G.
Bolton, Mr. and Mrs. Thomas C.
Borgese, Mrs. Thomas A.
Botkin, Dr. Daniel B.
Bowles, Mr. and Mrs. Francis P.
Bradley, Dr. and Mrs. Charles C.
Bronson, Mr. and Mrs. Samuel C.
Brown, Dr. and Mrs. Dugald E. S.
Brown, Dr. and Mrs. F. A., Jr.
Brown, Dr. and Mrs. Thornton
Buck, Mrs. John B.
Buffington, Mrs. Alice H.
Buffington, Mrs. George
Burger, Dr. and Mrs. Max M.
Burrell, Mrs. Peter E.
Burrough, Mrs. Arnold H.
Burt, Mr. and Mrs. Charles E.
Butler, Mr. and Mrs. Rhett W.
Butler, Mrs. E. G.
Calkins, Mr. and Mrs. G. N., Jr.
Campbell, Dr. and Mrs. David G.
Campbell, Mr. and Mrs. Worthington, Jr.
Capobianco, Mr. and Mrs. Pat J.
Carlson, Dr. and Mrs. Francis
Carlton, Mr. and Mrs. Winslow G.
Cashman, Mr. and Mrs. Eugene R.
Chambers, Dr. and Mrs. Edward L.
Cheney, Dr. Ralph H.
Claff, Mr. and Mrs. Mark
Clark, Mr. and Mrs. Hays
Clark, Mrs. James McC.
Clark, Dr. and Mrs. Leonard B.
Clark, Mr. and Mrs. LeRoy, Jr.
Clark, Mr. and Mrs. W. Van Alan
Clement, Dr. and Mrs. A. C.
Clowes Fund, Inc.
Clowes, Mr. Allen W.
Clowes, Dr. and Mrs. G. H. A., Jr.
Cohen, Dr. and Mrs. Seymour
Connell, Mr. and Mrs. W. J.
Cooper, Mr. and Mrs. John H., Jr.
Copeland, Mrs. D. Eugene
Copeland, Mr. and Mrs. Preston S.
Costello, Mrs. Donald P.
Crain, Mr. and Mrs. Melvin C.
Cramer, Mr. and Mrs. Ian D. W.
Crane, Mr. John
Crane, Josephine Foundation
Crane, Mrs. W. Carey
Cross, Mr. and Mrs. Norman C.
Crossley, Mr. and Mrs. Archibald M.
Crowell, Dr. and Mrs. Sears
Curtis, Dr. and Mrs. W. D.
Daignault, Mr. and Mrs. A. T.
Daniels, Mr. and Mrs. Bruce G.
Daniels, Mrs. F. Harold
Day, Mr. and Mrs. Pomeroy
Drummond, Mr. and Mrs. A. H., Jr.
DuBois, Dr. and Mrs. A. B.
Dunkerley, Mr. and Mrs. H. Gordon

DuPont, Mr. A. Felix, Jr.
Dyer, Mr. and Mrs. Arnold W.
Eastman, Mr. and Mrs. Charles E.
Ebert, Dr. and Mrs. James D.
Edwards, Dr. and Mrs. Robert L.
Egloff, Dr. and Mrs. F. R. L.
Elliott, Mrs. Alfred M.
Elsmith, Mrs. Dorothy O.
Epel, Mrs. David
Evans, Mr. and Mrs. Dudley
Ewing, Dr. and Mrs. Gifford C.
Fenno, Mrs. Edward N.
Ferguson, Dr. and Mrs. J. J., Jr.
Fine, Dr. and Mrs. Jacob
Firestone, Mr. and Mrs. Edwin
Fisher, Mrs. B. C.
Fisher, Mr. Frederick S., III
Fisher, Dr. and Mrs. Saul H.
Francis, Mr. and Mrs. Lewis W., Jr.
Fries, Dr. and Mrs. E. F. B.
Fye, Dr. and Mrs. Paul M.
Gabriel, Dr. and Mrs. Mordecai L.
Gaiser, Dr. and Mrs. David W.
Garfield, Miss Eleanor
Garrey, Dr. and Mrs. Walter
Gellis, Dr. and Mrs. Sydney
German, Dr. and Mrs. James L., III
Gifford, Mr. and Mrs. John A.
Gifford, Dr. and Mrs. Prosser
Gilbert, Dr. and Mrs. Daniel L.
Gilbert, Mrs. Helen H.
Gildea, Dr. Margaret C. L.
Gillette, Mr. and Mrs. Robert S.
Glass, Dr. and Mrs. H. Bentley
Glazebrook, Mrs. James R.
Glusman, Dr. and Mrs. Murray
Goldman, Dr. and Mrs. Allen S.
Goldring, Dr. Irene P.
Goldstein, Mrs. Moise H., Jr.
Grant, Dr. and Mrs. Philip
Grassle, Mr. and Mrs. J. K.
Green, Miss Gladys M.
Greene, Mr. and Mrs. William C.
Greer, Mr. and Mrs. W. H., Jr.
Greif, Dr. and Mrs. Roger L.
Grosch, Dr. and Mrs. Daniel S.
Gross, Mrs. Paul R.
Gruson, Mrs. Martha
Gunning, Mr. and Mrs. Robert
Haigh, Mr. and Mrs. Richard H.
Halvorson, Dr. and Mrs. Harlyn O.
Handler, Dr. and Mrs. Philip
Harvey, Dr. and Mrs. Richard B.
Haskins, Mrs. Caryl P.
Hassett, Dr. and Mrs. Charles
Hastings, Mrs. J. Woodland
Hawkins, Mr. Richard H., III
Heffron, Dr. and Mrs. Roderick
Henley, Dr. Catherine
Hiam, Mr. and Mrs. E. W.
Hiatt, Dr. and Mrs. Howard
Hibbard, Dr. Hope
Hill, Mrs. Samuel E.
Hilsinger, Mr. and Mrs. Arthur
Hirschfeld, Mrs. Nathan B.
Hobbie, Dr. and Mrs. John
Hocker, Mr. and Mrs. Lon
Hoffman, Rev. and Mrs. Charles
Horwitz, Dr. and Mrs. Norman H.
Hough, Mrs. George A., Jr.
Houston, Mr. and Mrs. Howard E.
Huettner, Dr. and Mrs. Robert
Hunziker, Mr. and Mrs. Herbert E.
Hutchinson, Mr. and Mrs. John
Hynes, Mr. and Mrs. Thomas J.
Inoué, Mrs. Shinya
Ireland, Mrs. Herbert A.
Issokson, Mr. and Mrs. Israel
Ivens, Dr. Sue
Jackson, Miss Elizabeth B.
Janney, Mrs. Wistar
Jewett, G. F., Foundation
Jewett, Mr. and Mrs. G. F., Jr.
Jones, Mr. and Mrs. Dewitt, III
Jones, Mr. and Mrs. Frederick, III
Jordan, Dr. and Mrs. Edwin P.
Kaan, Dr. Helen W.
Kahler, Mr. and Mrs. George A.
Kahler, Mrs. Robert W.
Kaminer, Mrs. Benjamin
Karush, Dr. and Mrs. Fred
Keith, Mr. Jean R.
Kelleher, Mr. and Mrs. Paul R.
Keosian, Mrs. Jessie
Kien, Mr. and Mrs. Pieter
Kingwell, The Rev. and Mrs. Wilbur J.
Kinnard, Mrs. L. Richard
Kivy, Dr. and Mrs. Peter
Koelsch, Mr. and Mrs. Herbert
Kohn, Dr. and Mrs. Henry I.
Koller, Dr. and Mrs. Lewis R.

Kuffler, Mrs. Stephen W.
Laderman, Mr. and Mrs. Ezra
Lash, Dr. and Mrs. James
Laster, Dr. and Mrs. Leonard
Laufer, Dr. and Mrs. Hans
Lawrence, Mr. Frederick V.
Lawrence, Mrs. William
Lazarow, Mrs. Arnold
Leatherbee, Mr. John A.
Lemann, Mrs. Lucy B.
Lenher, Mr. and Mrs. Samuel
Levine, Dr. and Mrs. Rachmiel
Leventhal, Ms. Monika Meyer
Lewis, Mr. T. Hohn
Lillie, Mrs. Karl C.
Lilly, Mr. and Mrs. Josiah K., III
Loeb, Mrs. Robert F.
Long, Mrs. G. C.
Lorand, Mrs. Laszlo
Lovell, Mr. and Mrs. Hollis R.
Lowengard, Mrs. Joseph
Lowe, Dr. and Mrs. Charles W.
Luria, Dr. and Mrs. S. E.
Mackey, Mr. and Mrs. William K.
MacLeish, Mrs. Margaret
MacNary, Mr. B. Glenn
MacNichol, Dr. and Mrs. Edward F., Jr.
Maher, Miss Anne Camille
Marks, Dr. and Mrs. Paul A.
Marsland, Dr. and Mrs. Douglas
Martyna, Mr. and Mrs. Joseph
Marvin, Dr. Dorothy H.
Maser, Dr. and Mrs. Morton
Mastroianni, Dr. and Mrs. L., Jr.
Mather, Mr. and Mrs. Frank J., III
Matthiessen, Mr. and Mrs. G. C.
Mavor, Mrs. James W., Sr.
McCusker, Mr. and Mrs. Paul T.
McElroy, Mrs. Nella W.
McLane, Mrs. T. Thorne
Meigs, Mr. and Mrs. Arthur
Meigs, Dr. and Mrs. J. Wister
Melillo, Dr. and Mrs. Jerry
The Mellon Foundation
Mellon, Mr. and Mrs. Richard P.
Metz, Mrs. Charles B.
Meyers, Mr. and Mrs. Richard
Miller, Dr. Daniel A.
Mixter, Mr. and Mrs. W. J., Jr.
Montgomery, Dr. and Mrs. Charles H.
Montgomery, Mr. and Mrs. Raymond B.
Moore, Mr. John W.
Morse, Mr. and Mrs. Charles L., Jr.
Morse, Mr. and Mrs. Richard S.
Moses, Mr. and Mrs. George L.
Moul, Mrs. Edwin T.
Neuberger, Mrs. Harry H.
Newton, C. H., Builders, Inc.
Nichols, Mrs. George
Nickerson, Mr. and Mrs. Frank L.
Norman, Mr. and Mrs. Andrew E.
Normandie Foundation
O'Herron, Mr. and Mrs. Jonathan
O'Sullivan, Dr. Renee Bennett
Olmsted, Mr. and Mrs. Christopher
Ortins, Mr. Armand
Pappas, Dr. and Mrs. George D.
Park, Mr. and Mrs. Franklin A.
Park, Mr. and Mrs. Malcolm S.
Parmenter, Miss Carolyn L.
Parmentier, Mr. George L.
Patten, Mrs. Bradley M.
Pecan, Ms. Erene V.
Pendergast, Mrs. Claudia
Pendelton, Dr. and Mrs. Murray E.
Pennington, Miss Anne H.
Perkins, Mr. and Mrs. Courtland D.
Person, Dr. and Mrs. Philip
Peterson, Mr. and Mrs. E. Gunnar
Peterson, Mr. and Mrs. E. Joel
Peterson, Mr. and Mrs. Raymond W.
Philippe, Mr. and Mrs. Pierre
Porter, Dr. and Mrs. Keith R.
Prosser, Mrs. C. Ladd
Putnam, Mr. Allan Ray
Putnam, Mr. and Mrs. W. A., III
Ratcliffe, Mr. Thomas G., Jr.
Raymond, Dr. and Mrs. Samuel
Read, Ms. Lee
Redfield, Dr. and Mrs. Alfred C.
Renek, Mr. and Mrs. Morris
Reynolds, Dr. and Mrs. George
Reynolds, Mr. and Mrs. James T.
Reznikoff, Dr. and Mrs. Paul
Ricca, Dr. and Mrs. Renato A.
Riggs, Mr. and Mrs. Lawrason, III

Riina, Mr. and Mrs. John R.
Robb, Ms. Alison A.
Robertson, Mrs. C. Stuart
Robertson, Dr. and Mrs. C. W.
Robinson, Dr. and Mrs. Denis M.
Rogers, Mrs. Julian
Root, Mrs. Walter S.
Ross, Mrs. John
Rowe, Mrs. William S.
Rubin, Dr. Joseph
Rugh, Mrs. Roberts
Russell, Mr. and Mrs. Henry D.
Ryder, Mr. and Mrs. Francis C.
Saunders, Dr. and Mrs. John W.
Saunders, Mrs. Lawrence
Savery, Mr. Roger
Sawyer, Mr. and Mrs. John E.
Schlesinger, Mrs. R. Walter
Scott, Mrs. George T.
Scott, Mrs. Norman E.
Sears, Mr. and Mrs. Harold B.
Segal, Dr. and Mrs. Sheldon
Shapiro, Mrs. Harriet S.
Shemin, Dr. and Mrs. David
Sheprow, Dr. and Mrs. David
Sherman, Dr. and Mrs. Irwin
Simkins, Mrs. Willard S.
Slater, Mr. David
Smith, Mr. and Mrs. Dietrich C.
Smith, Mrs. Homer P.
Smith, Mr. and Mrs. Robert I.
Smith, Mr. VanDorn C.
Snider, Mr. Eliot
Sonnebend, Mr. and Mrs. Paul
Specht, Mrs. Heinz
Steele, Mrs. M. Evelyn
Steinbach, Dr. and Mrs. H. B.
Stetten, Dr. and Mrs. DeWitt, Jr.
Stone, Dr. and Mrs. William
Stracher, Dr. and Mrs. Alfred
Stuart, Dr. Ann
Stunkard, Dr. Horace
Sturtevant, Mrs. A. H.
Swanson, Dr. and Mrs. Carl P.
Swope, Mr. and Mrs. Gerard L.
Swope, Mrs. Gerard, Jr.
Swope, Miss Henrietta H.
Tartakoff, Dr. Helen
Taylor, Dr. and Mrs. W. Randolph
Tietje, Mr. and Mrs. Emil D., Jr.
Tittler, Mrs. Sylvia
Todd, Mr. and Mrs. Gordon F.
Tolkan, Mr. and Mrs. Norman N.
Tompkins, Mrs. B. A.
Trager, Mrs. William
Troll, Dr. and Mrs. Walter
Tully, Mr. and Mrs. Gordon F.
Valois, Mr. and Mrs. John
Van Brunt, Mr. and Mrs. A. H., Jr.
Veeder, Mrs. Ronald A.
Vincent, Mrs. Helen J.
Waite, Mr. and Mrs. Charles E.
Waksman, Dr. and Mrs. Byron H.
Ware, Mr. and Mrs. J. Lindsay
Warren, Dr. and Mrs. Shields
Watt, Mr. and Mrs. John B.
Weisberg, Mr. and Mrs. Alfred M.
Wexler, Robert H., Foundation
Wheatley, Dr. Marjorie A.
Wheeler, Dr. and Mrs. Paul S.
Wheeler, Dr. and Mrs. Ralph E.
Whitney, Mr. and Mrs. Geoffrey G., Jr.
Wichterman, Dr. and Mrs. Ralph
Wickersham, Mr. and Mrs. A. A. Tilney
Wickersham, Mrs. James H., Jr.
Wilhelm, Dr. Hazel S.
Wilson, Mr. and Mrs. Robert E., Jr.
Witmer, Dr. and Mrs. Enos E.
Wolfinsohn, Mr. and Mrs. Wolfe
Woodwell, Mrs. George
Yntema, Dr. and Mrs. Chester L.
Zinn, Dr. and Mrs. Donald J.
Zipf, Dr. Elizabeth
Zwilling, Mrs. Edgar

III. CERTIFICATE OF ORGANIZATION

(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:-

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is THE MARINE BIOLOGICAL LABORATORY.

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirteenth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A.D. eighteen hundred and eighty-eight.

CHARLES ENDICOTT
Commissioner of Corporations)

IV. ARTICLES OF AMENDMENT

(On File in the Office of the Secretary of the Commonwealth)

We, James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meetings of the corporation:

VOTED: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

> "No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.
>
> "Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.
>
> "The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the Bylaws of the corporation, require action by the Corporate Members."

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of $10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

PAUL GUZZI
Secretary of the Commonwealth)

V. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 11, 1978)

I. (A) The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

(B) Marine Biological Laboratory admits students without regard to race, color, sex, national and ethnic origin to all the rights, privileges, programs and activities generally accorded or made available to students in its courses. It does not discriminate on the basis of race, color, sex, national and ethnic origin in employment, administration of its educational policies, admissions policies, scholarship and other programs.

II. (A) The members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees, upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by said Board of Trustees. Except as provided below, any Member may vote at any meeting, either in person or by proxy executed no more than six months prior to the date of such meeting. Members shall serve until their death or resignation unless earlier removed, with or without cause, by the affirmative vote of two-thirds of the Trustees then in office. Any Member who has attained the age of seventy years or has retired from his home institution shall automatically be designated a Life Member provided he signifies his wish to retain his membership. Life Members shall not have the right to vote and shall not be assessed for dues.

(B) The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

III. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk, elected or appointed by the Trustees as set forth in Article IX.

IV. The Annual Meeting of the Members shall be held on the Friday following the Second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 a.m. Subject to the provisions of Article VIII(2), at such meeting the Members shall choose by ballot six Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the Members may be called by the Chairman or Trustees to be held at such time and place as may be designated.

V. Twenty five Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person or by proxy at a meeting attended by a quorum (present in person or by proxy) shall constitute action on behalf of the Members.

VI. (A) Inasmuch as the time and place of the Annual Meeting of Members are fixed by these Bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of Members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least 15 days before such meeting, to each Member at his or her address as shown on the records of the Corporation.

(B) Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present or represented at the meeting, whether or not such Members constitute a quorum. It shall not be necessary to notify any Member of any adjournment.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated. Notice of Trustees' meetings may be given orally, by telephone, telegraph or in writing; and notice given in time to enable the Trustees to attend, or in any case notice sent by mail or telegraph to a Trustee's usual or last known place of residence, at least one week before the meeting shall be sufficient. Notice of a meeting need not be given to any Trustee if a written waiver of notice, executed by him before or after the meeting is filed with the records of the meeting, or if he shall attend the meeting without protesting prior thereto or at its commencement the lack of notice to him.

VIII. (A) There shall be four groups of Trustees:

(1) Trustees (the "Corporate Trustees") elected by the Members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees shall be divided into four classes of six, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms.

(2) Trustees ("Board Trustees") elected by the Trustees then in office according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Board Trustees shall be divided into four classes of three, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms. It is contemplated that, unless otherwise determined by the Trustees for good reason, Board Trustees shall be individuals who have not been considered for election as Corporate Trustees.

(3) Trustees ex officio, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(4) Trustees emeriti who shall include any Member who has attained the age of seventy years (or the age of sixty five and has retired from his home institution) and who has served a full elected term as a regular Trustee, provided he signifies his wish to serve the Laboratory in that capacity. Any Trustee who qualifies for emeritus status shall continue to serve as a regular Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Members or by the Board, as the case may be. The Trustees ex officio and emeriti shall have all the rights of the Trustees, except that Trustees emeriti shall not have the right to vote.

(B) The aggregate number of Corporate Trustees and Board Trustees elected in any year (excluding Trustees elected to fill vacancies which do not result from expiration of a term) shall not exceed nine. The number of Board Trustees so elected shall not exceed three and unless otherwise determined by vote of the Trustees, the number of Corporate Trustees so elected shall not exceed six.

(C) The Trustees and Officers shall hold their respective offices until their successors are chosen in their stead.

(D) Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee so to be removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him.

(E) Any vacancy in the number of Corporate Trustees, however, arising may be filled by the Trustees then in office unless and until filled by the Members at the next Annual Meeting. Any vacancy in the number of Board Trustees may be filled by the Trustees.

(F) A Corporate Trustee or a Board Trustee who has served an initial term of at least 2 years duration shall be eligible for re-election to a second term, but shall be ineligible for re-election to any subsequent term until two years have elapsed after he last served as a Trustee.

IX. (A) The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall annually elect a Treasurer who shall serve until his successor is selected and qualified. They shall elect a Clerk (a resident of Massachusetts) who shall serve for a term of 4 years. Eligibility for re-election shall be in accordance with the content of Article VIII (F) as applied to Corporate or Board Trustees. They shall elect Board Trustees as described in Article VIII (B). They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number as provided in Article X, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article X. They shall from time to time elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

(B) The Board of Trustees shall also have the power, by vote of a majority of the Trustees then in Office, to elect an Investment Committee and any other committee and, by like vote, to delegate thereto some or all of their powers except those which by law, the Articles of Organization or these Bylaws they are prohibited from delegating. The members of any such committee shall have such tenure and duties as the Trustees shall determine; provided that the Investment Committee, which shall oversee the management of the Corporation's endowment funds and marketable securities, shall include the Chairman of the Board of Trustees, the Treasurer of the Corporation, and the Chairman of the Corporation's Budget Committee, as *ex officio* members, together with such Trustees as may be required for not less than two-thirds of the Investment Committee to consist of Trustees. Except as otherwise provided by these Bylaws or determined by the Trustees, any such committee may make rules for the conduct of its business; but, unless otherwise provided by the Trustees or in such rules, its business shall be conducted as nearly as possible in the same manner as is provided by these Bylaws for the Trustees.

X. (A) The Executive Committee is hereby designated to consist of not more than ten members, including the ex officio Members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and the affirmative vote of a majority of those voting at any meeting at which a quorum is present shall constitute action on behalf of the Executive Committee. The Executive Committee shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by vote of the Board or by law. The Executive Committee may also appoint such committees, including persons who are not Trustees, as it may from time to time approve to make recommendations with respect to matters to be acted upon by the Executive Committee or the Board of Trustees.

(D) The Executive Committee shall keep appropriate minutes of its meetings and its action shall be reported to the Board of Trustees.

(E) The elected Members of the Executive Committee shall constitute as a standing "Committee for the Nomination of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

XI. A majority of the Trustees, the Executive Committee, or any other committee elected by the Trustees shall constitute a quorum; and a lesser number than a quorum may adjourn any meeting from time to time without further notice. At any meeting of the Trustees, the Executive Committee, or any other committee elected by the Trustees, the vote of a majority of those present, or such different vote as may be specified by law, the Articles of Organization or these Bylaws, shall be sufficient to take any action.

XII. Any action required or permitted to be taken at any meeting of the Trustees, the Executive Committee or any other committee elected by the Trustees as referred to under Article IX may be taken without a meeting if all of the Trustees or members of such committee, as the case may be, consent to the action in writing and such written consents are filed with the records of meetings. The Trustees or members of the Executive Committee or any other committee appointed by the Trustees may also participate in meeting by means of conference telephone, or otherwise take action in such a manner as may from time to time be permitted by law.

XIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees then in office.

XIV. These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend, or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of the meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Corporation Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

XV. The account of the Treasurer shall be audited annually by a certified public accountant.

XVI. The Corporation will indemnify every person who is or was a trustee, officer or employee of the Corporation or a person who provides services without compensation to an Employee Benefit Plan maintained by the Corporation, for any liability (including reasonable costs of defense and settlement) arising by reason of any act or omission affecting an Employee Benefit Plan maintained by the Corporation or affecting the participants or beneficiaries of such Plan, including without limitation any damages, civil penalty or excise tax imposed pursuant to the Employee Retirement Income Security Act of 1974; provided, (1) that the Act or omission shall have occurred in the course of the person's service as trustee or officer of the Corporation or within the scope of the employment of an employee of the Corporation or in connection with a service provided without compensation to an Employee Benefit Plan maintained by the Corporation, (2) that the Act or omission be in good faith as determined by the Corporation (whose determination made in good faith and not arbitrarily or capriciously shall be conclusive), and (3) that the Corporation's obligation hereunder shall be offset to the extent of any otherwise applicable insurance coverage, under a policy maintained by the Corporation or any other person, or other source of indemnification.

VI. REPORT OF THE DIRECTOR

"...There is no difficulty in forming government. It is not even a matter of choice whether there shall be one or not. Like breathing, it is not permitted to depend on our volition. Necessity will force it on all communities in some one form or another. Very different is the case as to constitution. Instead of a matter of necessity, it is one of the most difficult tasks imposed on man to form a constitution worthy of the name, while to form a perfect one—one that would completely counteract the tendency of government to oppression and abuse and hold it strictly to the great ends for which it is ordained—has thus far exceeded human wisdom, and possibly ever will."

—John C. Calhoun (1782–1850)
A Disquisition on Government

I have chosen this rubric, the product of an exceptional, though pessimistic mind among this nation's founding fathers, because I believe, as will emerge, that the most difficult task facing the Laboratory and those entrusted with its management is governance: its discussion, and, as may prove necessary, its modification.

Not long ago, survival itself was at issue. In those circumstances, the front line is always a battle-line. Economic survival, and the compromises thought by some to be necessary in aid of it, were the issues. The weapons of battle were budget-making, belt-tightening, a cold eye cast on proposals for spending: draconic measures to insure recovery, at least in part, of the costs of doing and teaching science. In that atmosphere, governance was, quite properly, seen as a merely peacetime concern, properly to be held in abeyance until the issue of battle became clear.

Remarkably, the issue *has*, in a short time, become clear. We have won the battle and are in the process of mopping up. The M.B.L. will survive, and it will continue into its second century as a beacon of biology. This is not to say that economic threat, to which so many sister institutions have succumbed, has vanished; rather, we have learned how to live with it, and there is no reason to believe that we shall need, in the forseeable future, to spend principal merely to stay at work. Our children will be able to come to the M.B.L. to partake, as we did, of its unique opportunity: the opportunity for those with imagination, energy, and self-discipline, independent of background or institution of origin, to work in close contact with, and to become, leaders of biology, pure and applied.

The tests we face for the immediate future are indeed tests, rather than battles. We will be tested in the responsibility to participate broadly and unselfishly, within the Corporation and the M.B.L. family generally, in the raising of what are for this place large sums of money—*not* for survival, but in order to insure that the full promise of this institution is realized. And we shall need to be flexible and wise about procedures and governance during the half-decade of transition ahead, in a period when our aging campus is returned to a condition matched to the quality of its scientific product, and during a time in which we attempt to endow the campus and its science financially, in such a way that survival will thereafter be assured. The choice of what to investigate, and what to teach, will thus be *ours*, not that of transient sponsors, nor of agencies of government, however benevolent.

PROGRAMS

1. *Summer Research.* In the summer of 1979 the M.B.L. was fully occupied. Every square foot of usable research space was rented. Upon the basis of reports made at the General Scientific Meetings, and of results I have myself heard about in other contexts, they were occupied with an unusual productivity. By virtue of an exceptional staff job of fitting people and programs into the available laboratories, and by a considerable effort of persuasion to secure agreements for sharing, it was

possible to accommodate all principal investigators who offered programs acceptable to our Research Space Committee, and to accommodate further the bulk of students and assistants for whom they sought places.

The summer of 1979 must therefore be counted as a very special one, even by the high standards of the M.B.L. In a time of increasingly restricted funding of individual research projects, and of research costs rising with awesome speed, there was sufficient recognition among productive investigators of the value of research at the M.B.L., and sufficient recognition of that value among their study section peers, to bring about full occupancy; to enlist some thousand people in the creation of the special scientific atmosphere that is a Woods Hole summer; and to limit disappointments and troubles to a gratifyingly small number.

The capstone of the research summer was the Friday Evening Lecture series, opened with an elegant presentation by Edward Wilson, and highlighted by a provocative discussion of biological "self" offered by the man who, better than any other, has chronicled the meaning of those lectures: Lewis Thomas. The session-closing General Scientific Meetings, revivified by the efforts of Hans Laufer a year earlier, fulfilled the promise of such a summer, under the direction of Joel Rosenbaum and his able associates.

Rather than make the Quixotic attempt to choose, among the nine or ten signal accomplishments of that summer, one or two for presentation in this report, I substitute an anecdote: In the early spring of 1980, sitting on a panel charged with the judgment and ranking of research proposals in the fields of my own work—cellular and developmental biology—I came across no fewer than five citations of results obtained in Woods Hole during the preceding summer as critical to the proposal substance. All those proposals were approved.

If ever there was a case of success breeding headaches, this is one. The indications for the summer of 1980 are more, and perhaps troublesomely more, of the same. At the time of writing the available space is not only fully claimed, but in a few cases doubly claimed. We have reached the practical limit of civilized space-sharing, and we have had an ominously *small* number of cancellations, by the standard of earlier years.

Clearly, an exciting and densely-populated summer is ahead of us in 1980. The new Parasitism course will be given for the first time; we can expect a distinguished addition, thereby, to the scientific population of Woods Hole. Rather than diminishing, the popularity and significance of the squid and its giant axon seem to be increasing. John Valois and his colleagues, quietly competent as they are, will have their work cut out for them, and the fishermen will come close inshore to compete. The question is: Can we, the Corporation, the Trustees, and the Administration, deal well and in a principled way with a situation foreign to our recent history (but quite familiar to our predecessors here): the need to turn away qualified applicants? We shall see. This resolves into one of those issues of governance for which I chose the rubric of this report.

Once again, the Friday Evening Lectures bid fair to be the intellectual and even the social highlight of the coming season. The speakers scheduled for it are of a level of recognition and achievement that can be matched by few institutions. The only mitigation I can think of, in the face of such disquieting and sequential eminence, is that every one of these speakers will, at some time during his stay in Woods Hole, need to answer some searching scientific questions.

2. *Year-round research.* The size of our year-round research program, as measured by numbers of persons engaged or the number of dollars spent on it, has again increased somewhat in the interval since the last Director's Report was written. In fiscal terms, this increase is a little faster, but marginally so, than inflation. In human terms, it is a good deal faster. The indications are, however, that by the fall of 1980 a quasi-equilibrium will have been established: retirements and space-constrictions (to the Library Readership that is the Nirvana of all devout M.B.L. scientists) will match nicely the establishment of new year-round laboratories. The undertaking

given to the Trustees in February of 1979 is to be upheld, *i.e.*, that consolidation, improvement, and growth of the year-round science at M.B.L. will not infringe *significantly*, over any stretch of a few years, upon the Laboratory's capacity to host its traditional transient programs.

The M.B.L. Ecosystems Center maintains its strength and a determined independence of its views. They and we look forward, mainly with happy anticipation, but in a minor way with intimations of hard work ahead, to the construction of our new Environmental Sciences Center, toward whose cost of approximately $1.2 million we received last year a grant of $0.9 million from the Fleischmann Foundation. The new facility will be a boon to all Woods Hole environmentalists.

The Laboratory of Biophysics, an intramural program of the National Institutes of Health here "on location," continues to function in a productive way and in a broad range of subdisciplines in neuroscience. The first of a new series of contracts between the old H.E.W. (now Department of Health and Human Resources) and the M.B.L., for the operation of this program, has been executed, and there is every reason to believe that the next five years will be as productive as the last, and—one hopes—somewhat easier to manage.

Dr. MacNichol's Sensory Physiology program continues to produce significant and technically admirable work, and to be supported for it, despite certain internecine conflicts within the cognizant advisory bodies on the question of the relevance of research on invertebrates. A small but well-deserved expansion of their space and facilities will take place shortly.

Cell Biology, that quintessential M.B.L. subject, and so ably represented on the campus in prior years, has had an upward shift coincident with the arrival here of Shinya Inoué as a year round investigator, and has been aided further by the establishment of Sidney Tamm as a faculty member of the B.U.M.P. program. Although Dr. Tamm has devised a number of wickedly effective methods for badgering the Director on matters of overhead, it is already clear that his work on cellular motility will flourish in Woods Hole.

The only year-round program, in fact, that has not shown growth consistent with last year's expectations is Developmental Biology, and this is directly traceable to the style of the Director's life, since he is a developmentalist (in principle), and was supposed to devote some reasonable part of his time to program-building in that important subject. To be sure, the Director's *own* scientific work has continued, thanks to generous arrangements made by the University of Rochester, and to the quality of his Rochester colleagues and students. It is however clear that some changes will have to be made in the Director's list of responsibilities so that he can establish and help to manage a new and major year-round research program.

3. *Education*. The Laboratory's educational programs are in good health, due, in part, to their inherent excellence, and in part to the nonthreatening, but conscious control and overview processes that have been instituted during the two years past.

The five January courses enrolled a total of 108 students, compared with 114 in 1978. This is a nonsignificant variation. The only systematic change suggested by a study of the January semester as a whole is that the quality of applicants rose detectably in 1979. Alan Fein, who had earlier served with Fred Lang as co-Instructor-in-Chief of the Neurobiology Course, took full responsibility for it less than three weeks before the start, following Fred Lang's tragic death in an automobile accident. Ted MacNichol, among others, stepped into the gap with courage and selflessness. The 1979 course was a great success, exceeded, perhaps, only by the success of the 1980 version, which had the benefit of a dedicated supervision by Daniel Alkon, one of the leaders of the M.B.L. year-round neuroscience program.

Among the short courses, the design and supervision of which are ably handled by Morton Maser, there was a high *esprit*, the best testament to which is the body of letters received from participants. A number of new courses were mounted in 1979, including Comparative Light and Electron Microscopy in Clinical Diagnosis (Harry Carter in charge), and Mariculture—the Culture of Marine Invertebrates for

Research Purposes (Carl Berg in charge). In 1979, nine short courses enrolled some 125 students, which approximates 87% of the planned capacity.

The summer teaching session, populated in 1979 by 142 students (compared with 148 in 1978), was an exciting one. Tom Humphreys and Joan Ruderman ended their tours of duty as co-directors of the Embryology Course, which had, if possible, an even more stellar student body than the year before; and Rudolf Raff, of the University of Indiana, took the initial steps in assuming directorship of the course for the term to come.

Six important and productive years of Jerome Schiff's directorship of the Botany course ended with this year's offering, and with an announced hiatus of one year in the offering of a marine botany course, during which a national committee of advisors will make proposals for the design of its successor. Dr. Lawrence Bogorad, the eminent Harvard botanist, will chair the committee of review, and will be in residence during the summer of 1980. A core group of the 1979 botany faculty will direct a specialized research and training program, under the rules for independent investigatorship, in the summer of 1980.

The Microbial Ecology course, directed by Harlyn Halvorson and Holger Jannasch, will be offered in an expanded version in 1980, residing temporarily, for that session, in the Loeb quarters normally used by Botany.

A term of outstanding service to the M.B.L.'s educational venture ended in 1979 with completion of Edward Kravitz's tour as instructor-in-chief of Neurobiology. He has been succeeded by John Hildebrand and Tom Reese. The identity and scientific reputations of those two insure that Dr. Kravitz's achievement will have the desired continuity.

Ronald Hoy accepted (generously) responsibility for the Neural Systems and Behavior course following a tragic death in the Gelperin family, the result of which was that the course's energetic founder, Alan Gelperin of Princeton, was unable to continue. Ronald Hoy's tenure will, undoubtedly, substitute for the Princetonian a certain Cornellian flavor to the enterprise, but that—however one judges Cornell as against Princeton—has already had the good effect of sharpening the substantive focus of the course, and of attracting to its important activities, at the interface between cellular neurobiology and behavioral science, a helpful amount of financial support.

The Boston University Marine Program, under the directorship of Arthur Humes, solidified its important position as an element of the Woods Hole educational spectrum. The tenth anniversary of the program was celebrated this year at an occasion of a very positive character, held in November at our Swope Center. Two new faculty have joined the program: Drs. Sidney Tamm and Christopher Price. C. K. Govind, of the University of Toronto, was a visiting senior faculty member during the second half of the year. There are now 34 B.U.M.P. graduate students in residence, about two-thirds of them candidates for the Ph.D. degree, supervised by five regular faculty who have the assistance of a much larger number of colleagues.

The level of B.U.M.P. activity in gestating and delivering Ph.D.'s, (in such fields as ecology, ecological genetics, physiology, and neurobiology) remains high—three the past year, and the production of quality research papers and of fruitful scientific interchanges, as, for example, in seminars, is equalled, and certainly not exceeded, by a very few other educational programs in marine biology.

Planning began, in 1979, for broadening in two directions of the activities now centered about the unique late-spring course in Aquatic Veterinary Medicine ("Aquavet"), which is coordinated by Donald Abt, supported in many ways by the parent schools of veterinary medicine (Pennsylvania and Cornell), and taught by a faculty group of multi-institutional origin. One direction will be research-centered, providing systematic opportunity for veterinarians in training and for other young biologists to carry out relevant research in M.B.L. laboratories. The other, an initiative that will take longer to be realized, but that will provide an important element of planning for the ultimate marine resources center and facilities, is the provision of diagnostic and other clinical services, under direction of qualified veterinarians, for the collection, maintenance, and use of marine animals.

4. *Scientific Meetings.* The skill with which Homer Smith and his staff manage the jigsaw puzzle of accommodations and facilities for scientific gatherings of various kinds received comment in the last report. Suffice it to say that in the past year—an even busier one than the preceding—there was no evidence of a decline in skill, nor of any likely reduction in the growth rate of M.B.L. as a desired location for serious scientific colloquy, especially in the quieter seasons.

The whole effort can be exemplified by the efficiency with which a threatening overload of participants in the symposium held in honor of Jim Ebert (September 11–13) was handled. Some who wanted to stay here during that time could not, but there were no angry nor wounded confrontations. Almost everyone seeking to participate was accommodated in one way or another (and as far away as the Plaza end of Falmouth), and, as the audience grew with each passing day from 200 to Standing Room Only in the Lillie Auditorium, everyone was fed, and had the opportunity to ask questions and to be heard. It is not surprising that the enthusiasm giving the event its ambience was reflected in major reports of it in the scientific press. The work of our Public Relations Office contributed importantly to the symposium's success.

In addition to the Ebert Symposium, the following (among many more not mentioned) were scientific gatherings held this year at the M.B.L.: The Society of General Physiologists, the Molecular Biophysics and Biochemistry Conference of Yale, a meeting on International Environmental Problems sponsored by the National Academy of Sciences and arranged by George Woodwell, a Harvard Medical School conference on Immunology, the Northeast Regional meeting of the Animal Behavior Society, a meeting of the National Foundation for Cancer Research; and a visit of Massachusetts Senator Paul Tsongas with leading Woods Hole environmental scientists.

While it is not true that these influxes of our slide-and-manuscript-bearing colleagues from the big cities disturb the peace, the beauty, and the historic somnolence of wintertime in Woods Hole, it *is* true that the possibility of escape, in this place, into an absence of thought, has come to be desperately small.

FUND-RAISING AND COST RECOVERY

Near the end of 1979, the Chairman of our Board of Trustees, Prosser Gifford, announced the Laboratory's new, very ambitious, and first fully-organized, long term fund-raising campaign, which has been named, for obvious reasons, the M.B.L. Second Century Fund. The goals and the strategy of that effort are too complex and too important to risk its misunderstanding by a mere summary in this limited space (over which the new Biological Bulletin editor, Charles B. Metz, will exert just as jealous a control as did his predecessor, "Gus" Russell-Hunter). Suffice it to say that in this year the M.B.L. mounted its first comprehensively planned capital campaign, and that as of the time of writing the totals are well ahead of the scheduled target. A full discussion of the Second Century Fund will be made available to the Corporation before the end of the 1980 summer session.

The cold numbers of the outcome are not without interest, however, and those I will communicate: In the year ending December, 1979, the M.B.L. actually received \$1,215,144 in gifts, and those receipts were underpinned by firm pledges of an additional \$1,985,500. The provenance of these gifts is as varied as the specific (or unrestricted) purposes for which they were given, but the happy implication of the totals will be evident to anyone who examines our financial statements for prior years. Details are available elsewhere in this Report.

Considerable attention has been given in the past year to the much-vexed question of overhead-cost recovery for research and educational programs. Vexed it is; more so than in colleges, universities, and full-time research laboratories, because of the peculiar mixture of activities carried on at the M.B.L. Of course, few non-profit institutions recover anything near the actual cost of mounting externally-sponsored research programs. The allowable charges for overhead are watched penuriously and renegotiated regularly by several agencies of government, all of which are rewarded for their effectiveness in saving money (which is as it should be). The M.B.L. recovers, unfortunately, a much smaller than average share of its costs. Among the reasons for this are (1) the space-rental method employed, which has built into it a required, but really inapplicable factor of "unused capacity" and a penalty based on it, (2) the danger that even if the rental rates were to be allowed to rise realistically, they would be too high for individual transient investigators to pay, (3) the fact that such payments are normally made as direct costs to research grants, while the parent institution recovers full, or nearly full indirect costs nevertheless, which is a form of double jeopardy, and (4) the fact that auditors and other contract officers of the granting agencies are poorly informed about the nature, scale, and value of M.B.L. operations. They have been inclined to deal with it as though it were a hospital or a kind of part-time college. Among the most troublesome consequences of the system and its inherent contradictions has been unpredictable change in rates from one year to the next, causing individual investigators much difficulty in the payment of bills.

Several good results have been obtained in the past year, in consequence of initiatives aimed at correcting the situation. The Directors of NIH and NSF have given strong and informed support to our brief to the auditors, seeking more realistic interim arrangements. The initiatives and that support have yielded the hoped-for relief in the form of an offical agreement on rental rates, covering a three-year period and taking account of inflation, and a reduction in the penalty for so-called "unused capacity." Negotiations with the financial officers of both our major granting agencies have, furthermore, brought about a significant broadening—and a mutual one—of understanding, particularly as regards the elements of unfairness that remain features of a system that treats year-round, in-house research grants in the same way as the funds supporting transients.

All parties to these negotiations have agreed that a new, simpler, fairer system of overhead recovery for the M.B.L. is to be worked out and presented for preliminary review before the end of the 1980 calendar year. The intention is to bring it into operation by the summer of 1981. Simultaneously, we are exploring, with cooperation of the agencies, forms of *general* support direct to the M.B.L., for the transient research and the educational programs of this Laboratory (and of the other U. S. marine laboratories), with a view to minimizing the direct cost burden on the individual transient research worker.

There is every reason to believe that the cost in time and effort now being paid by us for these initiatives will yield a very much larger return in the future. Our goals for that future are very simple: They are to recover the costs (and no more than the costs) of research and teaching done at the M.B.L. (assuming that the quality thereof will not decline from the present), *and* to make program quality, rather than the ability to pay rent, the essential determiner of participation.

MANAGEMENT

Biological Bulletin. Editorship of our journal has passed from the capable hands of W. D. Russell-Hunter to those of C. B. Metz. Russell-Hunter's was a distinguished tenure. He brought to the journal orderliness and efficiency that are rare among scientific journals in these times, and was able at the same time to impose upon it, without tyranny, something of the literacy and attention to detail that characterize his own writing. The Corporation owes him a debt of gratitude.

Charles Metz, in taking up the position's challenge, brings to it a broad experience in developmental and reproductive biology and in the management of educational enterprises. He intends to broaden the substantive range of *Biological Bulletin* articles, making the journal more representative of scientific interests of the Laboratory and its current programs. This, however, he hopes to accomplish without diminution of editorial rigor, nor a decline of the high standard of style set by his predecessor. He will be aided by several new and accomplished members of the editorial board. We wish him and his associates good luck, and set down here an undertaking already made *viva voce* by Corporation members who were consulted in the editorial succession: to help make the *Bulletin* a more representative vehicle, and a primary vehicle, for important papers in fields pursued at the M.B.L., while at the same time adhering to the policies that have made it an international journal of wide distribution, rather than a mere house publication.

The Board Chairman and The Treasurer. Drs. Prosser Gifford and Robert Mainer are very busy men. Each holds a demanding position and each gives time and interest to a broad range of educational and community enterprises. It was therefore with some concern that we began to seek, during 1979, a distinctly larger commitment of their time to M.B.L. management problems and to fund raising. They have responded magnanimously, and I cannot let the opportunity pass to thank them publicly, for the Trustees and for the Corporation as a whole. As must be evident, even from the skeletal indications of this Report, our management task has multiplied in difficulty and complexity. There is little of Parkinson's Effect (*i.e.*, the expansion of management to fill all available space and budgets) in this. By the applicable standards, M.B.L.'s management team is very small for the size and complexity of the enterprise. This has been true in the past, and it is more strikingly true now. Yet the place *has* changed, as have the times and the requirements for getting support and employing people. A large part of our management team is volunteer: the time-consuming and difficult work of the Executive Committee, for example, is done without compensation for its members. The Board Chairman and the Treasurer, who are two of the members, have a particularly heavy burden of work. We have added to it by broadening the participation of Trustees in such important decisions as fund-raising initiatives, capital equipment and resources acquisitions, property management, and salary-setting. Drs. Gifford and Mainer have accepted these new responsibilities with good cheer and met them with skill. There is no way that we can pay them for the valuable time they have given, and will, we hope, continue to give, except by our honest gratitude.

New Positions. The Controller and the General Manager will shortly have the assistance of a skilled personnel officer, who will relieve them of the responsibilities they now bear for personnel management, while at the same time collaborating with them and with the Director in relevant new policy-making. This is a step long in review and long needed, particularly because however decent and familial our employer-employee relations have been (and they have been unusually so), the law now requires attention to record-keeping, to reporting, and to pension matters at a level of detail that cannot be managed by any one person part time.

In addition, the search has begun for a senior officer, at the level of Assistant Director, who will have broad responsibility in general administration and in the Laboratory's Planning and Development activities. This is a position whose creation is indispensable for a successful outcome of long-term plans for campus rehabilitation and for enlarging the endowment. The Laboratory has received a significant grant that will underwrite the costs of this office for the first few years, during which time its performance and contribution will be observed with care.

Committees. In that context the Executive Committee and the administration have begun to plan for a regular and orderly process of review, in which administrators *and* their positions will be subject to a study of performance, in quite the same way as staff scientists and support staff (and professors in universities) have their performances reviewed.

It is our hope in the coming year to enlist a much closer involvement of all Trustees, and of Corporation members generally, in such processes. A deliberate and thorough review of membership rotation for the standing committees was begun early in 1979. It continues to make visible and useful progress. The work of the Library Committee is particularly worthy of mention in this regard. Under the Chairmanship of Edward Adelberg, this broadly-based committee has made a study of a perennial trouble-subject: the management of our superb Library. The final report to the Corporation is due at the end of the 1980 summer session, but interim reports, combined with a new and heartening spirit of cooperation among the senior administrators of this Laboratory and of the Woods Hole Oceanographic Institution, have already produced important agreements. Among them is a fairer distribution of effort and budgetary support for the Library than ever before.

It is now clear that the final Library proposals will entail no threatening discontinuities of style or services, but that they will at the same time result in a signal improvement of the Library's holdings, specialized services, and capability for attracting external support.

CONCLUSION

I return, finally, to the broader issues of constitution and government with which this report began. We have made every effort to control the changes taking place in the M.B.L.'s size, range of programs, and facilities, in such a way as to minimize (although, unfortunately, not to eliminate) dislocations and inconvenience. The directions of change have prior Corporation agreement: rehabilitation of the campus, so as to fit it for the kinds of research and teaching now done here (and to be done here in the future); the addition of certain new facilities, such as the much-needed marine resources center, the environmental sciences center, and a carefully planned expansion of housing; consolidation and strengthening of year-round programs within the context of the M.B.L. program as a whole, and without long-term infringement of the transient programs; reorganized management of endowment and properties. What has not, and necessarily not, been argued in nearly so much detail is the machinery of governance by which these changes are to be brought about. John Calhoun's wry but perfectly accurate assessment of the central issue applies: government (or management) there must be, but the rules for it, or the constitution, must have an inherent flexibility sufficient to deal with change *and* to prevent tyranny. Even so the rules must be changed from time to time.

This is hardly the place for a listing of the issues of governance about which we shall have to confer, since a report is, after all, concerned with past and present, rather than with the future. An indication of the nature of those issues would not, however, be inappropriate. We need a reexamination of the role played by our Trustees, and particularly by the Board Trustees. We need some new understandings, if not more rules, about administration generally: how big it needs to be and ought to be, for this unique institution, and how its quality, whatever the agreed size, is to be maintained. We need understandings as to how the Laboratory's scientific programs are to be kept under some reasonable central control, as is demanded increasingly by granting agencies, while at the same time insisting that administration exists to serve the programs, rather than the reverse.

The changes required for the M.B.L.'s survival in strength into its second century are large. Let us hope that an equally large recommitment to watchfulness, to participation, *and* to good will can be made this year by the Corporation membership and, indeed, by all friends of the Laboratory, as the countdown to our second century begins.

VII. TREASURER'S REPORT

The Laboratory achieved its 1979 financial objective of maintaining parity between revenues and expenses. In fact, a modest surplus of $31,000 was recorded—a gratifying improvement over the operating deficits of 1978 and 1977. However, this result does not include provision for depreciation on the Laboratory's physical plant and equipment, about which I shall have more to say in my concluding comments.

Aggregate revenues of $4,766,000 in 1979 were $509,000 greater than in the prior year. Among the more important contributors to the increase are the following:

—$140,000 in additional laboratory fees and overhead recoveries. A significant factor here was a 13 percent increase in the approved overhead rate for federal grants and contracts ($30.50 in 1978 to $34.60 in 1979).
—$74,000 in additional total investment income, partly resulting from higher rates on short-term investments and partly from improved returns on endowment and quasi-endowment funds which, since February, 1979, have been under full-time professional supervision.
—$443,000 in additional federal funding of direct costs of research performed under grants and contracts.

Offsetting a portion of the above increases was a decline in unrestricted gifts—$115,000 in 1979 versus $241,000 in 1978. However, gift income tends to be unpredictably variable. For example, 1978 saw the receipt of a gift of real estate and an unusually large foundation gift. As we build the momentum of the Laboratory's Second Century Fund campaign, we are likely to see even greater variability in unrestricted gifts, both because some longtime contributors will temporarily shift their attention to the Laboratory's capital needs and because the campaign may elicit a certain number of gifts from donors who prefer not to contribute to "bricks and mortar."

Aggregate expenses increased $442,000 in 1979, reaching a total of $4,735,000. Much of this resulted from the flow-through of expenses associated with the higher level of federal contract research noted in the discussion of income. Inflation's shadow darkened virtually every category of costs in 1979 and glooms the coming months even more. I am impressed by the performance of the Laboratory's administration and staff in controlling expenses, often achieved by uncommon ingenuity in the use of limited resources.

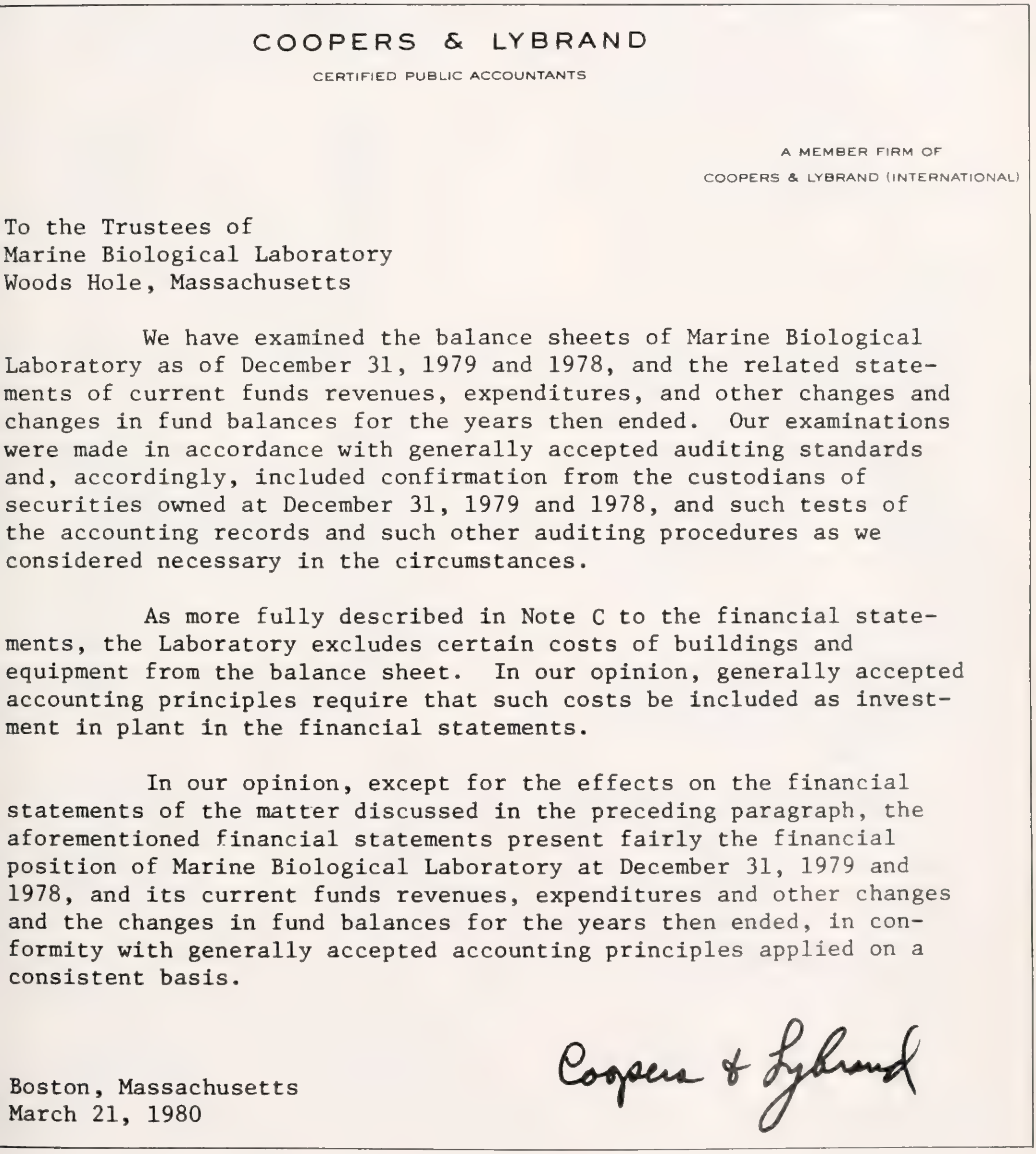
During 1979, the Woods Hole Oceanographic Institution and the Marine Biological Laboratory reached agreement on a formula for sharing joint library costs. As a result, we now have a more logical and predictable method for reimbursement of the costs of acquiring and managing library resources for the joint benefit of WHOI and MBL.

Also during 1979, the MBL negotiated a fixed three-year (1980–82) overhead rate with its government auditors. The three-year period, in contrast to the former year-to-year approvals, enables investigators to more accurately budget for multi-year research projects and permits the Laboratory to better estimate its income.

The Laboratory's 1980 budget aims at an objective of $69,000 of revenues in excess of expenses, before provision for depreciation. Its achievement will be difficult. In addition to inflation's toll, the Laboratory will face the added expenses of the steps being taken to assure its future. In particular, monies must be spent now to organize and conduct the substantial fund raising effort to rehabilitate an aging physical plant, add facilities and increase endowment.

This brings me to the point I must make about the Laboratory's long standing practice of not funding the depreciation of its physical assets. It is tempting to conclude that the Laboratory is doing well if its revenues cover cash outlays. Such a conclusion brings an illusory comfort. Depreciation, although a non-cash expense, reflects the loss in value of assets attributable to deterioration and obsolescence. Moreover, in inflationary periods, stated depreciation underestimates the cost of replacing assets acquired or built during earlier times.

The alternative to the funding of depreciation assuming we are not willing to permit catabolic processes to overtake the Laboratory's viability is to dedicate ourselves to periodic aggressive fund raising to generate the means by which the MBL's physical plant can be maintained, repaired, rehabilitated or, in some cases, replaced. It is for this reason that the recently announced Second Century campaign is vitally important and deserving of the help of everyone associated with the Laboratory.



COOPERS & LYBRAND
CERTIFIED PUBLIC ACCOUNTANTS

A MEMBER FIRM OF
COOPERS & LYBRAND (INTERNATIONAL)

To the Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts

We have examined the balance sheets of Marine Biological Laboratory as of December 31, 1979 and 1978, and the related statements of current funds revenues, expenditures, and other changes and changes in fund balances for the years then ended. Our examinations were made in accordance with generally accepted auditing standards and, accordingly, included confirmation from the custodians of securities owned at December 31, 1979 and 1978, and such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances.

As more fully described in Note C to the financial statements, the Laboratory excludes certain costs of buildings and equipment from the balance sheet. In our opinion, generally accepted accounting principles require that such costs be included as investment in plant in the financial statements.

In our opinion, except for the effects on the financial statements of the matter discussed in the preceding paragraph, the aforementioned financial statements present fairly the financial position of Marine Biological Laboratory at December 31, 1979 and 1978, and its current funds revenues, expenditures and other changes and the changes in fund balances for the years then ended, in conformity with generally accepted accounting principles applied on a consistent basis.

Coopers & Lybrand

Boston, Massachusetts
March 21, 1980

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1979 and 1978

Assets	*1979*	*1978*
Current Funds:		
Unrestricted:		
Cash, including deposits at interest	$ 424,763	$ 382,396
Money market securities	500,000	—
Accounts receivable, net of allowance for uncollectible accounts of $1,716 in 1979 and $4,500 in 1978	858,466	699,708
Other assets	11,186	15,367
Due to restricted current funds	(729,275)	(278,075)
Due (to) from invested funds	(8,151)	103,248
Total unrestricted	1,056,989	922,644
Restricted:		
Cash	12,285	18,973
Investments, at cost; market value: 1979—$1,881,590; 1978—$1,297,551 (Notes B and F)	1,900,292	1,297,530
Due from unrestricted current fund	729,275	278,075
Due from invested funds	350,967	260,967
Total restricted	2,992,819	1,855,545
Total current funds	$ 4,049,808	$ 2,778,189
Invested Funds:		
Cash	5,589	6,982
Investments, at cost; market value: 1979—$4,250,838; 1978—$3,950,184 (Notes B and F)	3,936,137	4,084,003
Due (to) from unrestricted current fund	8,151	(103,248)
Due to restricted current funds	(350,967)	(260,967)
Total invested funds	$ 3,598,910	$ 3,726,770
Plant Fund:		
Land, buildings and equipment at cost (Note C)	12,258,651	12,421,929
Less accumulated depreciation	4,251,598	4,060,407
Total plant fund	$ 8,007,053	$ 8,361,522

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1979 and 1978

Liabilities and Fund Balances	*1979*	*1978*
Current Funds:		
Unrestricted:		
Accounts payable and accrued expenses	$ 268,369	$ 316,657
Deferred income	68,006	61,432
Fund balance	720,614	544,555
Total unrestricted	1,056,989	922,644
Restricted:		
Fund balances:		
Unexpended gifts and grants	2,897,679	1,777,677
Unexpended income of endowment funds	95,140	77,868
Total restricted	2,992,819	1,855,545
Total current funds	$4,049,808	$2,778,189
Invested Funds:		
Endowment funds	1,918,170	2,174,027
Quasi-endowment funds	934,143	934,143
Retirement fund (Note D)	746,597	618,600
Total invested funds	$3,598,910	$3,726,770
Plant Fund:		
Invested in plant	8,007,053	8,361,522
Total plant fund	$8,007,053	$8,361,522

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF CURRENT FUNDS REVENUES, EXPENDITURES, AND OTHER CHANGES

for the years ended December 31, 1979 and 1978

	Unrestricted		*Restricted*		*Total*	
	1979	*1978*	*1979*	*1978*	*1979*	*1978*
Revenues:						
Instruction:						
Tuition	$ 238,378	$ 236,391	$ 15,500	$ 43,100	$ 253,878	$ 279,491
Grants and contracts:						
Government	19,788	7,360	215,719	215,759	235,507	223,119
Private			53,887	70,446	53,887	70,446
Research:						
Laboratory rentals	565,631	503,576			565,631	503,576
Grants and contracts:						
Government	283,770	206,741	1,304,680	861,515	1,588,450	1,068,256
Private	79,468	90,550	225,887	290,402	305,355	380,952
Dormitory	319,010	320,020			319,010	320,020
Dining Hall	179,507	157,270			179,507	157,270
Library	130,609	135,028			130,609	135,028
Biological Bulletin	87,416	86,171			87,416	86,171
Support departments:						
Research services	220,914	187,741			220,914	187,741
Marine resources	92,277	79,822			92,277	79,822
Investment income	171,215	159,703	188,641	141,707	359,856	301,410
Gifts	114,520	240,563	120,178	132,864	234,698	373,427
Other	139,491	91,141			139,491	91,141
Total revenues	2,641,994	2,502,077	2,124,492	1,755,793	4,766,486	4,257,870

	Unrestricted		Restricted		Total	
	1979	1978	1979	1978	1979	1978
Operating expenditures:						
Instruction	214,687	194,993	132,387	219,587	347,074	414,580
Research	(7,019)	3,813	1,675,172	1,264,631	1,668,153	1,268,444
Scholarships and stipends			236,958	216,958	236,958	216,958
Dormitory	143,794	135,769			143,794	135,769
Dining Hall	169,604	149,249			169,604	149,249
Library	252,713	235,401	36,089	22,757	288,802	258,158
Biological Bulletin	84,082	87,273			84,082	87,273
Support departments:						
Research services	355,719	354,485	5,000	25,400	360,719	379,885
Marine resources	226,690	218,794		34	226,690	218,828
Administration	567,396	514,204	36,314	4,263	603,710	518,467
Plant operation	602,969	643,550	2,572	2,163	605,541	645,713
Total expenditures	2,610,635	2,537,531	2,124,492	1,755,793	4,735,127	4,293,324
Excess (deficit) of revenues	31,359	(35,454)	—	—	31,359	(35,454)
Transfers and additions:						
Excess of restricted gifts, grants and investment income received over amounts expended			1,137,274	188,173	1,137,274	188,173
From plant fund—proceeds of sale of plant assets	144,700				144,700	
To plant fund—donated unrestricted real estate		(47,342)				(47,342)
Net transfers and additions	144,700	(47,342)	1,137,274	188,173	1,281,974	140,831
Net increase (decrease) in fund balances	$ 176,059	$ (82,796)	$1,137,274	$ 188,173	$1,313,333	$ 105,377

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF CHANGES IN FUND BALANCES

for the years ended December 31, 1979 and 1978

	Current Funds		Invested Funds			
1978	*Unrestricted*	*Restricted*	*Endowment*	*Quasi-Endowment*	*Retirement*	*Plant Fund*
Balances at December 31, 1977	$ 627,351	$ 1,667,372	$2,172,160	$934,143	$540,202	$8,596,042
Increases:						
Unrestricted current fund revenues	2,502,077					
Grants and gifts		2,054,971				
Realized net gains on sale of investments			1,867			
Investment income		150,546			32,013	
Addition to pension fund					65,573	
Tuition		43,100				
Decreases:						
Instruction, research and general expenditures	(2,537,531)	(1,755,793)				
Indirect costs		(304,651)				
Payments to pensioners					(19,188)	
Depreciation						(281,862)
Transfers—additions (deductions):						
Transfer to plant fund	(47,342)					47,342
Balances at December 31, 1978	544,555	1,855,545	2,174,027	934,143	618,600	8,361,522

1979	Current Funds		Invested Funds			Plant Fund
	Unrestricted	*Restricted*	*Endowment*	*Quasi-Endowment*	*Retirement*	
Increases						
Unrestricted current fund revenues	2,641,994					
Grants and gifts		3,422,789				
Investment income		206,503			38,148	
Proceeds of sale of equipment						144,700
Addition to pension fund					113,062	
Tuition		15,500				
Decreases:						
Instruction, research and general expenditures	(2,610,635)	(2,124,492)				
Indirect costs		(383,026)				
Realized net losses on sale of investments			(255,857)			
Payments to pensioners					(23,213)	
Net book value of equipment sold						(76,455)
Depreciation						(278,014)
Transfers—additions (deductions):						
Proceeds of sale of equipment	144,700					(144,700)
Balances at December 31, 1979	$ 720,614	$ 2,992,819	$1,918,170	$934,143	$746,597	$8,007,053

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

Notes to Financial Statements

A. *Purpose of the Laboratory:*

The purpose of Marine Biological Laboratory (the "Laboratory") is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

B. *Significant Accounting Policies:*

Basis of Presentation—Fund Accounting

In order to ensure observance of limitations and restrictions placed on the use of resources available to the Laboratory, the accounts of the Laboratory are maintained in accordance with the principles of "fund accounting." This is the procedure by which resources are classified into separate funds in accordance with activities or objectives specified. In the accompanying financial statements, funds that have similar characteristics have been combined.

Externally restricted funds may only be utilized in accordance with the purposes established by the source of such funds. However, the Laboratory retains full control over the utilization of unrestricted funds. Restricted gifts, grants, and other restricted resources are accounted for in the appropriate restricted funds. Restricted current funds are reported as revenue when expended for current operating purposes. Unrestricted revenue is reported as revenue in the unrestricted current fund when received.

Endowment funds are subject to restrictions requiring that the principal be invested and only the income utilized. Quasi-endowment funds have been established by the Laboratory for the same purposes as endowment funds, however, any portion of these funds may be expended. Certain accounts for the prior year have been reclassified to the current reporting format.

Investments

Investments purchased by the Laboratory are carried at cost. Investments donated to the Laboratory are carried at fair market value at date received. For determination of gain or loss upon disposal, cost is determined based on the specific identification method.

Investment Income and Distribution

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

Investment income includes income from the investments of specific funds and from the pooled investment account. Income from the pooled investment account is distributed to the participating funds on the basis of the market value at the beginning of the quarter, adjusted for the cost of any additions or disposals during the quarter.

C. *Land, Buildings and Equipment:*

Following is a summary of the plant fund assets:

Classification	*1979*	*1978*
Land	$ 639,693	$ 639,693
Buildings	10,190,430	10,190,430
Equipment	1,428,528	1,591,806
	12,258,651	12,421,929
Less accumulated depreciation	4,251,598	4,060,407
	$ 8,007,053	$ 8,361,522

The original cost of land, buildings and related initial furnishing equipment is capitalized when the assets are acquired. The cost of subsequent additions and purchases, repairs and remodeling is expensed when incurred. Equipment and remodeling expenditures amounted to approximately $76,000 and $125,000 in 1979 and 1978, respectively.

Depreciation is computed using the straight-line method over estimated useful lives of 40 years for buildings and 20 years for equipment.

D. *Retirement Fund:*

The Laboratory has a noncontributory pension plan for substantially all full-time employees which complies with the requirements of the Employee Retirement Income Security Act of 1974. The actuarially determined pension expenses charged to operations in 1979 and 1978 were $81,892 and $65,673, respectively. The Laboratory's policy is to fund pension costs accrued.

E. *Pledges and Grants:*

As of December 31, 1979 and 1978, the following amounts remain to be received from previous gifts and grants for specific research and instruction programs, and are expected to be received as follows:

	December 31, 1979		*December 31, 1978*	
	Unrestricted	*Restricted*	*Unrestricted*	*Restricted*
1979			$34,666	$2,161,535
1980	$ 50,500	$3,689,270		28,000
1981	49,000	215,000		
1982	15,000			
	$114,500	$3,904,270	$34,666	$2,189,535

F. *Investments:*

The following is a summary of the cost and market value of investment assets at December 31, 1979 and 1978, and the related investment income and disposition of investment income for the years ended December 31, 1979 and 1978.

	Cost		*Market*		*Investment Income*	
	1979	*1978*	*1979*	*1978*	*1979*	*1978*
Invested Funds:						
U. S. Government securities	$ 986,397	$ 610,661	$ 930,834	$ 594,205	$ 88,030	$ 35,718
Corporate fixed income obligations	1,162,164	689,185	1,038,929	583,572	64,683	42,969
Common stocks	1,705,817	2,080,664	2,213,951	2,123,631	142,530	131,333
Preferred stocks	64,210	83,032	49,575	54,548	2,976	3,654
Units in combined pension fund	—	602,912	—	576,679	(1,719)	33,114
Real estate (market at cost)	17,549	17,549	17,549	17,549		
Total	$3,936,137	$4,084,003	$4,250,838	$3,950,184	$296,500	$246,788
Less custodian fees					23,794	14,867
					272,706	231,921

	Cost		*Market*		*Investment Income*	
	1979	*1978*	*1979*	*1978*	*1979*	*1978*
Current Restricted Funds:						
U. S. Government securities	$1,900,292	$1,297,530	$1,881,590	$1,297,551	118,707	86,807
Current Unrestricted Funds:						
Earned on corporate savings accounts and money market funds					24,453	23,534
Investment income					$415,866	$342,262
Disposition of Investment Income:						
Restricted for current use:						
Utilized in current operations					188,641	141,707
Available for future operations					17,862	8,839
Total restricted current funds					206,503	150,546
Retirement fund					38,148	32,013
Unrestricted—utilized in current operations					171,215	159,703
					$415,866	$342,262

VIII. REPORT OF THE LIBRARIAN

The Library received a marvelous gift of $450,000 from the Andrew W. Mellon Foundation! Relief is now in sight for our never-ending space problem, as much of this money is earmarked for renovation for the Library section of the Lillie Building. Plans are underway to move all the Administration offices to the Candle House within the next year or so, and the present space occupied by these offices will become part of the Library. Tentative plans call for the removal of the Reprint Collection from the basement stack to a section of the main administration area thereby releasing this lower stack for expansion of the journal collection. Eventually the book section on Stack Three will be moved to the third floor, (where a section of the book collection is already housed) so that ultimately all five stacks will hold the entire journal collection, from A to Z.

Agreements were reached with the Woods Hole Oceanographic Institution involving joint cost-sharing arrangements for: 1) approximately 500 journal subscriptions; 2) staff salaries; 3) major reference collections of interest to both institutions; 4) space costs; 5) physical renovations. This was a major move toward a joint cooperative effort involving both MBL and WHOI in maintaining and expanding the Library in the years to come.

A Library questionnaire was prepared with the help of many users and the members of the Library Committee. This was mailed at the end of December to 2,400 past and present users of the Library. The results will be tabulated and published in the 1980 report.

IX. EDUCATIONAL PROGRAMS

SUMMER

Embryology

Instructors-in-chief

Humphreys, Tom D., University of Hawaii
Ruderman, Joan V., Harvard Medical School

Other faculty, staff, and lecturers

Bender, W., California Institute of Technology
Botstein, D., Massachusetts Institute of Technology
Brandhorst, B., McGill University, Canada
Crain, W., Worcester Foundation for Experimental Biology
Crawford, William, Stanford University
De Robertis, E., Cambridge University, England
Dolecki, Gregory J., University of Hawaii
Efstratiadis, A., Harvard Medical School
Ernst, V., Massachusetts Institute of Technology
Fregien, Nevis, University of Hawaii
Gross, Paul, Marine Biological Laboratory
Hahn, W., University of California
Hereford, L., Brandeis University
Hunt, T., Cambridge University, Cambridge, England
Kedes, L., Stanford University
Lodish, H., Massachusetts Institute of Technology
Newrock, K., McGill University, Canada

RAFF, R., Indiana University
REEDER, R., Hutchinson Cancer Center
RICE, DOUGLAS, University of Hawaii
ROSBASH, M., Brandeis University
ROSENTHAL, ERIC, Harvard Medical School
RUBIN, G., Harvard Medical School
SCHMIDT, LYN, Harvard Medical School
SOUTHERN, E., University of Edinburgh, Edinburgh, Scotland
TANSEY, TERESE, Harvard Medical School
VILLA-KOMAROFF, L., University of Massachusetts, Worcester
WEINTRAUB, H., Hutchinson Cancer Center
WHITTAKER, J. R., Wistar Institute, Philadelphia
WINKLER, M., University of California, Berkeley
ZACKS, SUSAN, Providence, Rhode Island
ZIFF, E., Rockefeller University

Students[1]

*BAKER, ELLEN, Wesleyan University
*BALLINGER, DENNIS, Massachusetts Institute of Technology
*BANNON, GARY, Iowa State University
*BOARDMAN, MARK, University of Hawaii, Kewalo Marine Laboratory
*BUDORICK, NANCY, University of Illinois, Chicago
*DAWKINS, BEVERLY, Atlanta University
*DEMARCHEZ, MICHEL, University of Grenoble, France
*DEUTSCH, STEPHEN, California State University, Long Beach
*DURICA, DAVID, Worcester Foundation for Experimental Biology
*EARL, CHRISTOPHER, Stanford University
*HAMMOND, MARTIN, University of Washington
*HASTINGS, KENNETH, University of Virginia
*HOROVITCH, SHARON, Massachusetts Institute of Technology
*KOLEGA, JOHN, Yale University
*LEVER, PEGGY, University of Connecticut
*MCLANE, JOHN, University of Rhode Island
*MICKEL, FRANCES, University of North Carolina
*MURRAY, ANDREW, Harvard University
*PETERSON, J. SCOTT, University of Texas
*PHILLIPS, WILLIAM, University of Virginia
*SCHLOSS, JEFFERY, Yale University
*SERUNIAN, LESLIE, Harvard University
*SMITH, JONATHAN, Harvard Medical School
*SMOUSE, DAVID, Stanford University
*WINKLER, MATTHEW, University of California, Berkeley
*YONEMOTO, WES, SUNY at Stony Brook

EXPERIMENTAL MARINE BOTANY

Instructor-in-chief

SCHIFF, JEROME A., Brandeis University

Other faculty, staff, and lecturers

AHMADJIAN, VERNON, Clark University
ALBERTE, RANDALL S., University of Chicago

---

[1] All summer students listed completed the formal course programs. Asterisk indicates those completing post-course research sessions.

Coleman, Annette, Brown University
Ellis, Richard, Bucknell University
Fiore, James, Suffolk University
Gaffron, Hans, University of Florida (retired)
Gibson, Jane, Cornell University
Goldfine, Steven, SUNY at Stony Brook
Guillard, Robert L., Woods Hole Oceanographic Institution
Loewus, Frank A., Washington State University
Lyman, Harvard, SUNY at Stony Brook
Mauzerall, David, Rockefeller University
McCandless, Esther L., McMaster University
Price, Carl, Rutgers University
Quatrano, Ralph S., Oregon State University
Schatz, Scott, University of Massachusetts
Sears, James R., Southeastern Massachusetts University
Stephens, Raymond E., Marine Biological Laboratory
Stern, Arthur I., University of Massachusetts
Troxler, Robert, Boston University
Wilce, Robert, University of Massachusetts

Students[1]

Christianson, John, University of Bridgeport
Egan, Kathleen, Southern Illinois University
*Frey, Marica, Oberlin College
Friedman, Alan, Humboldt State University
*Gustafson, Darel, University of Bridgeport
Hayhome, Barbara, University of Nebraska
Hicks, Heraline, Atlanta University
*Hogan, Arthur, University of Rochester
Klarer, Constance, DePauw University
*Kursar, Thomas, University of Chicago
*Mazzella, Lucia, Stazione Zoologica di Napoli, Italy
Patrick, Jane, University of Louisville
*Penncavage, Nancy, SUNY at Stony Brook
Rudnick, Marla, Yale University
*Squier, Thomas, Oberlin College
*Thaler, David, University of Massachusetts, Boston

MARINE ECOLOGY

Instructors-in-chief

Teal, John M., Woods Hole Oceanographic Institution
Valiela, Ivan, Boston University/Marine Biological Laboratory

Other faculty, staff, and lecturers

Cappuzo, Judith, Woods Hole Oceanographic Institution
Cole, Timothy J., Boston University/Marine Biological Laboratory
Cowles, Timothy, Woods Hole Oceanographic Institution
Gieselman, Joy, Woods Hole Oceanographic Institution
Grassle, J. Frederick, Woods Hole Oceanographic Institution
Hobbie, John, Marine Biological Laboratory
Howarth, Robert, Marine Biological Laboratory
Jackson, Jeremy B. C., Johns Hopkins University
Jannasch, Holger, Woods Hole Oceanographic Institution
Jefferies, Robert L., University of Toronto

Madin, Larry, Woods Hole Oceanographic Institution
Mann, Roger, Woods Hole Oceanographic Institution
Morris, James, Marine Biological Laboratory
Odum, William, University of Virginia
Paine, Robert, University of Washington
Peterson, Bruce, Marine Biological Laboratory
Peterson, Susan, Woods Hole Oceanographic Institution
Sanders, Howard, Woods Hole Oceanographic Institution
Steele, John, Woods Hole Oceanographic Institution
Tenore, Kenneth, Skidaway Institute of Oceanography
Van Raalte, Charlene, Hampshire College
Van Raalte, Christopher, Hampshire College
Werme, Christine, Boston University/Marine Biological Laboratory
Wiebe, Peter, Woods Hole Oceanographic Institution
Wiltse, Wendy, Woods Hole Oceanographic Institution

Students[1]

Anderson, Leslie, Oberlin College
*Blackwell, Katherine, Oberlin College
*Boynton, John
*Chen, Peter, Stanford University
*Conde, Jesus, Gran Mariscal de Ayacucho, Venezuela
*Delsing, Selma, Caribbean Marine Biological Institute
*Ford, Timothy, Sussex University, England
*Fulton, Laurie, Hampshire College
*Gale, Judith, University of Massachusetts, Boston
Kiselica, Daria, Seton Hill College
*Kreitler, Virginia, University of Pennsylvania
*McHenry, Thomas, Yale University
*McKinney, Laurie, Stephens College
*Meissinger, Joyce, University of California, San Diego
*Mercer, Daniel, University of Texas, Austin
*Olson, Deanna, University of California, San Diego
*Paton, David, Edgartown, Massachusetts
*Phillips, Neal, University of Georgia
*Pourreau, Catherine, College of Wooster
*Shelton, Mark, Colorado State University
Thomas, William, University of California, San Diego

Microbial Ecology

Instructors-in-chief

Halverson, Harlyn O., Brandeis University
Jannasch, Holger W., Woods Hole Oceanographic Institution

Other faculty, staff, and lecturers

Alexander, Martin, Cornell University
Atwood, Kimball C., Columbia University
Cuhel, Russell L., Woods Hole Oceanographic Institution
Dastoor, Vernour, National Aeronautics and Space Administration
Davis, Bernard D., Harvard University
Demain, Arnold L., Massachusetts Institute of Technology
Desmaris, David J., Ames Laboratories, Moffet Field
Farrington, John W., Woods Hole Oceanographic Institution
Gibson, Jane A., Cornell University

GOLDMAN, JOEL C., Woods Hole Oceanographic Institution
HANSON, RICHARD S., University of Wisconsin
HASTINGS, J. WOODLAND, Harvard University
HOLLAND, HEINRICH D., Harvard University
KEOSIAN, JOHN, Marine Biological Laboratory
KORNBERG, HANS, Cambridge University, England
LANYI, YANOS, Ames Laboratories, Moffet Field
LEADBETTER, EDWARD R., University of Connecticut
MAHLER, HENRY R., Indiana University
MARGULIS, LYNN, Boston University
ORÓ, JOHN, University of Houston
PECK, HARRY D., University of Georgia
POINDEXTER, JEANNE S., Public Health Research Institute, New York
RICH, ALEX, Massachusetts Institute of Technology
RICH, JOSIAH D., Columbia University
SAGER, RUTH, Harvard University
SCHLESINGER, WALTER R., Rutgers University
STANIER, ROGER Y., Institut Pasteur, France
TAYLOR, CRAIG D., Woods Hole Oceanographic Institution
VALIELA, IVAN, Marine Biological Laboratory
VAN HOLDE, KENSAL E., Oregon State University
VARON, MAZAL, The Hebrew University, Jerusalem, Israel
VINCENT, WALTER S., University of Delaware
WOESE, CARL, University of Illinois, Urbana
WALTER, JAMES, Arecibo Laboratories, Puerto Rico
WILSON, EDWARD O., Harvard University
WOODWELL, GEORGE M., Marine Biological Laboratory

Students[1]

AALSETH, CAROLE, Virginia Polytechnic Institute and State University
*ASHENDORF, DEBRA, Boston University
FACHON, SUZANNE, University of Vermont
*KELLER, JANIS, University of New Hampshire
*KUSERK, FRANK T., Moravian College
*MILLS, DAVID, University College of North Wales, United Kingdom
*UHLINGER, DAVID J., University of Nebraska
*VALERA, FRANCISCO, Colegio Universitario, Alicante, Spain
WILLIAMS, BETSY L., University of Washington

NEUROBIOLOGY

Instructor-in-chief

KRAVITZ, EDWARD A., Harvard Medical School

Other faculty, staff, and lecturers

ARMSTRONG, C., University of Pennsylvania
BATTELLE, B., National Institutes of Health
BIZZI, E., Massachusetts Institute of Technology
COHEN, JONATHAN B., Harvard Medical School
FINKELSTEIN, A., Albert Einstein University
FISCHBACH, GERALD D., Harvard Medical School
FURSHPAN, EDWIN J., Harvard Medical School
GAINER, H., National Institutes of Health
GESCHWIND, N., Harvard Medical School
GLUSMAN, S., Harvard Medical School
GOY, M., Harvard Medical School

Guthrie, M., Committee to Combat Huntingtons Disease
Hall, Linda, Massachusetts Institute of Technology
Harris-Warrick, R., Harvard Medical School
Herbert, E., University of Oregon
Hildebrand, John G., Harvard Medical School
Hubel, D. H., Harvard Medical School
Humphreys, T., University of Hawaii
LaFratta, J., Harvard Medical School
Landis, Dennis, Harvard Medical School
Landis, Story C., Harvard Medical School
Lasek, R., Case Western Reserve University
Lester, H., California Institute of Technology
Llinas, R., New York University
MacLeish, Peter R., Harvard Medical School
Matsumoto, S., Harvard Medical School
Neubig, R., Harvard Medical School
O'Lague, Paul, University of California, Los Angeles
Ornberg, R., National Institutes of Health
Pollard, H., National Institutes of Health
Potter, Camilla, Swarthmore College
Potter, David D., Harvard Medical School
Rahamimoff, R., Hebrew University, Jerusalem
Raviola, E., Harvard Medical School
Reese, B., National Institutes of Health
Reese, T. S., National Institutes of Health
Rheuben, M., Pennsylvania State University
Rose, B., University of Miami Medical School
Salpeter, M., Cornell University
Schroeder, B., National Institutes of Health
Shotton, D., Imperial College, London
Siegel, Ruth, Harvard Medical School
Suga, N., Washington University, St. Louis
Wasserman, J., Pennsylvania State College
Wexler, N., NINCDS

Students[1]

*Anderson, David J., Rockefeller University
*Davis, Miriam R., Princeton University
*Hurwitz, Jerard, Albert Einstein College of Medicine
*Mearow, Karen, McMaster University, Canada
*Nerbonne, Jeanne M., California Institute of Technology
*Noble, Mark D., University College London, England
*Ratner, Nancy, Indiana University
*Sarabhai, Anand, Biocenter Laboratory, Ahmedabad, India
*Smith, Sharon W., Stanford University
*Wehner, Jeanne M., Dight Institute for Human Genetics
*Wiley, Ronald G., Cornell Medical College
*Zigmond, Michael J., University of Pittsburgh

PHYSIOLOGY

Instructor-in-chief

Van Holde, Kensal, Oregon State University

Other faculty, staff, and lecturers

Ackers, Gary, Johns Hopkins University
Angeles, Leticia, University of Philippines System

Astrin, Susan, Institute for Cancer Research
Begg, David A., Harvard Medical School
Brenowitz, Michael, Duke University Marine Laboratory
Brown, Jay, University of Virginia
Christopher, Al, Loyola University
Cohen, William, Hunter College
Dentler, Bill, University of Kansas
Elgin, Sarah, C. R., Harvard University
Engelhard, V. H., Harvard University
Galvin, Patrick, University of Colorado, Denver
Goldman, Robert, Carnegie-Mellon University
Hamkalo, Barbara, University of California, Irvine
Hepler, P. K., University of Massachusetts, Amherst
Howe, Chris, Yale University
Huang, Ru-Chih, Johns Hopkins University
Hutchinson, Nancy, University of California
Huxley, H. E., Cambridge, England
Inoué, Shinya, Marine Biological Laboratory
King, Jonathan, Massachusetts Institute of Technology
McCarthy, Bob, Dartmouth College
Miller, Molly, Swarthmore College
Mooseker, Mark, Yale University
Pollard, H. B., National Institutes of Health
Reeder, Ronald H., Hutchinson Cancer Center
Rosa, Margaret, Yale University
Rosenbaum, Joel, Yale University
Selick, Barry, University of Pennsylvania
Stephens, Raymond, Marine Biological Laboratory
Summers, Jesse, Institute for Cancer Research
Szent-Gyorgyi, Andrew, Brandeis University
Von Hippel, Peter, University of Oregon
Weber, Anne Marie, University of Pennsylvania
Weintraub, Harold, Hutchinson Cancer Center

Students[1]

*Ausio, Juan C., Escuela Tecnica Superior, Barcelona, Spain
Bell, Jerry A., Simmons College
*Blanchard, Edward, Jr., University of Cincinnati
Buas, Michael, National Cancer Institute
Bynum, Robert D., Dartmouth College
*Davis, Lisa M., University of North Carolina
Deatly, Anne, Rockefeller University
*Dorflinger, Laneta, Yale University
*Greenberg, Sharon, Cornell University
*Hamblin, Martha T., Oregon State University
*Heilmann, Larry, Wesleyan University
Howard, Elizabeth Ann, University of California, Berkeley
Johnson, Carl Hirschie, Stanford University
*Kaznowski, Christine E., University of California, Irvine
*Kelly, Charles M., Case Western Reserve University
*Kew, David, Wesleyan University
Lewis, Larry, Millersville State College
*May, Gregory, Yale University
*Mercer, Robert, Syracuse University
*Miller, Carol Lynn, University of Pennsylvania
Panitz, Polly, Oberlin College

Paton, Arthur, University of Tennessee
Richardson, Marge, Iowa State University
Rigney, David, Institute for Cancer Research
*Smith, Denice, Dartmouth College
Suprynowicz, Frank, University of California, Berkeley
Tartakoff, Alan, University of Geneva, Switzerland
*Thompson, John, University of Massachusetts
*Vierling, Elizabeth, University of Chicago
*Vinson, Charles, University of Virginia
*Walsh, Jennifer, University of Montana
Wang, Lei-Lei, Baylor College of Medicine
Wong, Yuk-Chor, Harvard University
*Wood, Periann, Wesleyan University
*Yager, Thomas, University of Denver

NEURAL SYSTEMS AND BEHAVIOR

Acting instructor-in-chief

Hoy, Ronald R., Cornell University

Other faculty, staff, and lecturers

Bennett, Michael, Albert Einstein College of Medicine
Carew, T., Columbia University
Crane, Linda, Rockefeller University
Eisner, Thomas, Cornell University
Evans, Charles, Columbia University
Fraser, J., Brandeis University
Gibson, Dan, Boston University
Gould, J., Princeton University
Hertel, Horst, Free University, Berlin
Kalmijn, Adrianus J., Woods Hole Oceanographic Institution
Konishi, Mazakazu, California Institute of Technology
Levinthal, Cyrus, Columbia University
Macagno, Eduardo, Columbia University
Menzel, Randolf, Free University, Berlin
Muller, K., Carnegie Institute
Nelson, Margaret, Harvard University
Nicholls, John, Stanford University
Nottebohm, Fernando, Rockefeller University
Palka, John, University of Washington
Pearson, Keir, University of Alberta, Canada
Prior, D., University of Kentucky
Purpura, Keith, Columbia University
Reingold, Stephen, Princeton University
Simmons, James, Washington University
Suga, Nobuo, Washington University
Suh, Dick Y., Columbia University
Suh, Kyungsun, Columbia University

Students[1]

*Bablanian, Gayne M., University of Rhode Island
*Burke, Bryant, Yale University
*Cotran, Paul, Harvard University
*Debski, Elizabeth, University of Virginia
Derby, Charles, Boston University

*Farley, Joseph, Princeton University
*Flanagan, Thomas R., Wesleyan University
Frost, William, Princeton University
Hartzog, Grady, III, University of Florida
Hoffmann, Robert, New York University
Homberg, Uwe, Free University, Berlin
Jurman, Mark, University of Pennsylvania
Lamour, Yvon, National Institutes of Health
Levitt, Tami, Harvard School of Public Health
Morita, Elaine, Stanford University
Parikh, Jitendra, Physical Research Laboratory, Ahmedabad, India
Presti, David, California Institute of Technology
*Schaner, Pamela J., Pennsylvania State University
*Schneider, Jill, Wesleyan University
*Stewart, Randall R., Texas Tech University
Takahashi, Terry, SUNY Downstate Medical Center
*Teague, Barbara, Arizona State University
Walthall, Walter, Boston University

---

[1] All summer students listed completed the formal course programs. Asterisk indicates those completing post-course research sessions.

JANUARY

Behavior

Instructor-in-chief

Atema, Jelle, Boston University/Marine Biological Laboratory

Other faculty, staff, and lecturers

Alkon, Daniel, National Institutes of Health/Marine Biological Laboratory
Ashkenas, Linda, Boston University
Barlow, Robert, Syracuse University
Berg, Carl J., Jr., Harvard University/Marine Biological Laboratory
Bowles, Francis, Marine Biological Laboratory
Brisbin, I. Lehr, Savannah River Ecology Program
Capranica, Robert, Cornell University
Carey, Francis, Woods Hole Oceanographic Institution
Crow, Terry, National Institutes of Health/Marine Biological Laboratory
Dethier, Vincent, University of Massachusetts
Douglas, Matthew, Boston University
Elgin, Randall, Boston University
Goodenough, Judith, University of Massachusetts
Griffin, Donald, Rockefeller University
Harrington, Jonathan, SUNY at Stony Brook
Kalmijn, Adrianus J., Woods Hole Oceanographic Institution
Kanwisher, John, Woods Hole Oceanographic Institution
Karnofsky, Elisa, Marine Biological Laboratory
Kreithen, Mel, Cornell University
Levine, Joseph, Harvard University
Michel, Celia, Newton, Massachusetts
Michel, George, Newton, Massachusetts
Muller-Schwarze, Dietland, SUNY at Syracuse
Nelson, Margaret, Harvard Medical School
Payne, Katy, Woods Hole, Massachusetts
Smith, Doug, SUNY at Stony Brook
Stenzler, Dan, Setauket, New York

STUART, ALASTAIR, University of Massachusetts
SULZMAN, FRANK, Harvard Medical School
SWAIN, TONY, Boston University
WATKINS, BILL, Woods Hole Oceanographic Institution
WILLIAMS, JANET, Swarthmore College
WILLIAMS, TIMOTHY, Swarthmore College

Students

BAGBY, BRENDA, Oberlin College
BROYLES, JUDY, University of Texas, Arlington
BRY, JOHN, Tufts University
COWPERTHWAITE, Leslie, Massachusetts Audubon
DAVIS, ROBERT, Bennington College
DEVINE, DANA, Boston University
DONALDSON, TERRY, University of Guam
EDDS, PEGGY, University of Maryland
EPPI, RENE, SUNY at Stony Brook
FREDERICK, PETER, Hockessin, Delaware
GIBBONS, SHELLEY, Tufts University
GOEBEL, ANNA, Abilene Christian University
JESWINE, NAINA, University of Kansas
KENDALL, SCOTT, University of Iowa
KUZMA, GREGORY, University of California, Riverside
MCCORMICK, FRANK, St. Louis University
MCFARREN, MARTHA, University of Vermont
NONACS, PETER, University of Kentucky
OWEN, RALPH, University of Massachusetts
REILLY, PAMELA, College of St. Catherine
RITTENHOUSE, ANN, Boston University
SPEARS, JONATHON, University of Delaware
TROTT, THOMAS, Boston University

COMPARATIVE PATHOLOGY OF MARINE INVERTEBRATES

Instructor-in-chief

BANG, FREDERIK B., Johns Hopkins University

Other faculty, staff, and lecturers

BANG, BETSY G., Johns Hopkins University
COOPER-WILLIS, ANN, Johns Hopkins University
EDDS, KENNETH, Marine Biological Laboratory
FARLEY, C. AUSTIN, NOAA Marine Fisheries Laboratory
JOHNSON, PHYLLIS, NOAA Marine Fisheries Laboratory
PEARCE, JACK, NOAA Marine Fisheries Laboratory
MICHELSON, E., Harvard School of Public Health
PRENDERGAST, ROBERT, Johns Hopkins University
REINISCH, CAROL, Harvard University
RICKLES, FRED, University of Connecticut Health Center

Students

ANDREWS, CHARLA, Johns Hopkins University
BENNETT, HOLLIS, Battelle Memorial Institute
COOK, MALTHA, Sloan-Kettering Institute
COX, FANETHIA, Texas Southern University

ELSTON, RALPH, Cornell University
FONT, WILLIAM, University of Wisconsin
HARTLEY, DEAN, University of Illinois
HAZELL, RHONDA, College of St. Elizabeth
MCCORMICK, STEPHEN, Massachusetts Institute of Technology
MEYERS, THEODORE, Cornell University
MOORE, CAROL, Northeastern University
PATTERSON, MICHAEL, University of Washington
PETERS, ESTHER, University of South Florida
SUTTON, MARILYN, Atlanta University
SWANSON, DONALD, Columbia University

DEVELOPMENTAL BIOLOGY

Instructor-in-chief

VINCENT, WALTER S., University of Delaware

Other faculty, staff, and lecturers

BELL, EUGENE, Massachusetts Institute of Technology
COLLIER, JACK, Brooklyn College
DETHIER, VINCENT, University of Massachusetts
EDDS, KENNETH, Marine Biological Laboratory
GERBI, SUSAN, Brown University
GOLDMAN, ALLEN, University of Pennsylvania
GROSS, PAUL R., Marine Biological Laboratory
HALVORSON, HARLYN O., Brandeis University
INOUÉ, SHINYA, University of Pennsylvania, Marine Biological Laboratory
LASH, JAY, University of Pennsylvania
LAUFER, HANS, University of Connecticut
MARCUS, NANCY, Woods Hole Oceanographic Institution
MASER, MORTON D, Marine Biological Laboratory
MASSOVER, WILLIAM, Brown University
MCGLYNN, Clare, Wheaton College
MILLER, RICHARD, Temple University
MOSS, DAVID, University of Delaware
SKINNER, DOROTHY, Oak Ridge National Laboratories
STEPHENS, RAYMOND, Marine Biological Laboratory
TASCA, RICHARD, University of Delaware
BARBARA WRIGHT, Boston Cancer Institute

Students

BACKSTROM, LEEANN, College of the Holy Cross
BARNUM, SUSAN, Central Michigan University
BUMILLER, MARK, Tulane University
CANGEMI, PHYLLIS, Columbia University
COLLINS, ERICA, CUNY, Medgar Evers College
CROWLEY, PAULA, Skidmore College
DEPELTEAU, AUDREY, Rensselaer Polytechnic Institute
DUBE, FRANCOIS, University of Montreal, Canada
ERICKSON, MOIRA, Sweetbriar College
HANKE, JEFFREY, College of St. Rose
KANDERS, BONNIE, Cornell University
LATINWO, LEKAN, Alabama A&M University
LEWIS, ANDREA, Atlanta University
LOWREY, DAVID, Macalester College

LUTZ, DOUGLAS, University of Manitoba, Canada
NAZAR, NANCY, Monmouth College
ORTIQUE, DENISE, Dillard University
PAGE, JENNIFER, Tulane University
PILLSBURY, KATHERINE, Middlebury College
SLITER, TIMOTHY, Harvard University
SPIETH, TIMOTHY, CUNY at Genesee
SULLIVAN, ROBERT, University of Montreal, Canada
WILSON, NANCY, Macalester College

ECOLOGY

Instructor-in-chief

WOODWELL, GEORGE M., Marine Biological Laboratory

Other faculty, staff, and lecturers

BERWICK, S., Yale University
BOWLES, FRANCIS, Marine Biological Laboratory
BURROUGHS, R. H., Marine Biological Laboratory
CAPRONICA, ROBERT, Cornell University
DEWAR, R., University of Connecticut
EMERY, K. O., Woods Hole Oceanographic Institution
HOBBIE, JOHN E., Marine Biological Laboratory
HOUGHTON, R. A., Marine Biological Laboratory
HOWARTH, R., Marine Biological Laboratory
JEFFRIES, R., University of Toronto, Canada
JORDAN, M. J., Woods Hole, Massachusetts
LOVEJOY, T. E., World Wildlife Fund
MARGULIS, L., Boston University
MELILLO, J. M., Marine Biological Laboratory
MOORE, B. N., University of New Hamshire
MORRIS, JAMES T., Marine Biological Laboratory
NAIMAN, R., Woods Hole Oceanographic Institution
PETERSON, B. J., Marine Biological Laboratory
REMINGTON, C., Yale University
RICHARDS, A., Yale University
ROWE, G., Woods Hole Oceanographic Institution
SMITH, F. E., Harvard University
SOLBRIG, O., Harvard University
SZENT-GYORGYI, ALBERT, Marine Biological Laboratory
TEAL, JOHN M., Woods Hole Oceanographic Institution
VALIELA, IVAN, Boston University/Marine Biological Laboratory

Students

AKROYD, ROBERT E., College of Santa Fe
APTHORP, LUCY, Mount Holyoke College
BROWN, BETSY, University of Delaware
COOPER, JAY, Washington College
COUGHLIN, JOHN, Assumption College
DEEGAN, LINDA, University of New Hampshire
EMBERTON, KENNETH, Ohio University
FRANKLIN, WENDY, Cape Cod Planning & Economic Development Commission
GEORGE, ALICE, Oberlin College
GOLDOWITZ, BETH, University of California, Davis
HAIRE, PAUL, Brown University

KANE, ANN, Rutgers University
LULL, WENDY, University of New Hampshire
MANNING, JOHN, Worcester Polytechnic Institute
MARTYN, KATHLEEN, University of Wisconsin, Green Bay
MOIR, RONALD, Antioch, New England
SALTMARSH, JOHN, University of Massachusetts
SHUTTLEWORTH, JANE, Hampshire College
STANTON, SUSAN, Hampshire College
SUDOL-JOY, JAMES, University of Wisconsin, Green Bay
SYMMES, FREDERICK, Hampshire College
ZIMMERMAN, KAREN, Colorado State University

NEUROBIOLOGY

Instructors-in-chief

FEIN, ALAN, Marine Biological Laboratory
MACNICHOL, EDWARD F., JR., Marine Biological Laboratory

Other faculty, staff, and lecturers

ADELMAN, WILLIAM, National Institutes of Health
ATEMA, JELLE, Boston University/Marine Biological Laboratory
BARLOW, ROBERT, Syracuse University
CAPRANICA, ROBERT, Cornell University
CAREW, TOM, Columbia University
COHEN, MELVIN, Yale University
COSTELLO, WALTER, Yale University
DETHIER, VINCENT, University of Massachusetts
FRANK, ERIC, Harvard Medical School
FRAZIER, DONALD, University of Kentucky
FRITZ, LAWRENCE, Rockefeller University
FURSHPAN, EDWIN J., Harvard Medical School
GOLDMAN, DAVID, Harvard Medical School
HAROSI, FERENC, Marine Biological Laboratory
KALMIJN, ADRIANUS J., Woods Hole Oceanographic Institution
KAPLAN, EHUD, Rockefeller University
KRAVITZ, EDWARD, Harvard Medical School
LENT, CHUCK, Brown University
LEVAY, SIMON, Harvard Medical School
MACLEISH, PETER, Harvard Medical School
MARSHALL, LARRY, Harvard Medical School
MAURO, ALEX, Rockefeller University
PAYNE, KATY, Woods Hole, Massachusetts
RUBIN, LEE, Harvard Medical School
SHASHOUA, VICTOR, Harvard Medical School
STUART, ANN, Harvard Medical School
WYMAN, ROBERT, Yale University

Students

BEAUDRY, MICHEL, University of Sherbrooke
BERGERON, BRYAN, Tulane University
BRUNKEN, WILLIAM, SUNY at Stony Brook
CALLE, PAUL, University of Pennsylvania
CURTISS, WILLIAM, Oberlin College
DERBY, CHARLES, Boston University/Marine Biological Laboratory
FRASER, JEAN, Brandeis University

HADLOCK, ROBIN, University of Rhode Island
HARDY, MATTHEW, Oberlin College
JONES, JOSEPH, College of Saint Rose
KENNEDY, DEBBIE, College of Saint Rose
LISCUM, LAURA, Columbia University
LUM, JANET, University of Texas
LUQUE, ANTONIO, University of Utah
MINK, JONATHAN, Wesleyan University
MORTON, HOLMES, Trinity College
PFORTMILLER, GARY, Benedictine College
PIEKOS, WALTER, Clemson University
PUBLICOVER, NELSON, McGill University, Canada
REGAN, LAURA, Swarthmore College
ROUTHOUSKA, GLENN, Eisenhower College
TANK, DAVID, Cornell University
TIPPERMAN, RICHARD, Hamilton College
WALTHALL, WALTER, Boston University/Marine Biological Laboratory

SHORT COURSES

FREEZE–ETCHING IN ELECTRON MICROSCOPY

Instructor-in-chief

STEERE, RUSSELL, U. S. Department of Agriculture

Other faculty, staff, and lecturers

ERBE, ERIC, U. S. Department of Agriculture
MOSELY, MIKE, U. S. Department of Agriculture
SCARBOROUGH, ANN, Louisiana State University
SLADOVICH, HEDY, Marine Biological Laboratory
SOMMER, JOACHIM R., Duke University

Students

BUCANA, CORAZON, Frederick Cancer Research Center
CRAFT, JOSEPH, Massachusetts Eye and Ear Infirmary
CRAWFORD, BRUCE, University of British Columbia, Canada
CURTIS, JOSEPH, Clark University
GRUBER, SUE ELLEN, Mount Holyoke College
JONES, BETTY RUTH, Morehouse College
KAZNOWSKI, CHRISTINE E., University of California, Irvine
LAI, YIN-LOK, Yale University
LEWIS, BARBARA A., Yale University
MORRIS, GERALD, Queen's University, Canada
NEWCOMB, WILLIAM, Queen's University, Canada
WENK, EUGENE J., New York Medical College
UNGAR, FLORETTE, Columbia University

BIOLOGICAL ELECTRON MICROSCOPY FOR TECHNICIANS

Instructor-in-chief

MASER, MORTON D, Marine Biological Laboratory

Other faculty, staff, and lecturers

SCARBOROUGH, ANN, Louisiana State University
COPELAND, D. EUGENE, Marine Biological Laboratory

Sladovich, Hedy, Marine Biological Laboratory
Waterbury, John, Woods Hole Oceanographic Institution

Students

Channer, Judith, Montefiore Hospital
Cubberley, Virginia, New York University
Egan, Sheila, Massachusetts General Hospital
Farmer, Carol Lee, Armed Forces Radiobiology Research Institute
Goodman, Steven, Marine Biological Laboratory
Justis, C. Sue, Miami University
Lewis, Carrie M., Bigelow Laboratory for Ocean Sciences
Reese, William, Naval Dental Research Institute
Riesco, Maria Beatriz, Center for Energy and Environmental Research
Rosenheimer, Julie, University of Wisconsin
Shiffer, Kathleen A., Milton S. Hershey Medical Center
Trofatter, Lorraine, Ethicon Research Foundation

Scanning Electron Microscopy in the Biological Sciences

Instructor-in-chief

Wetzel, Bruce, National Cancer Institute

Other faculty, staff, and lecturers

Albrecht, Ralph, University of Wisconsin
Archuletta, Michael D., ETEC Corporation
Corbey, Kathleen, Tousimis Research Corporation
Houghton, Susan, Woods Hole Oceanographic Institution
Kendig, Esther, National Institutes of Health
Laudate, Anthony, JEOL-USA, Inc.
Lunn, Ron, Perkin-Elmer Corporation
Scarborough, Ann., Louisiana State University
Tousimis, A. J., Tousimis Research Corporation

Students

Blake, James A., University of the Pacific
Brittain, Faith, W. L. Gore & Associates
Gibson, Robert A., Harbor Branch Foundation
Hartwig, Virginia M., Armed Forces Radiobiology Research Institute
Jamuar, Mahendra P., Brookhaven National Laboratory
Keller, Katharyn L., Chicago Medical School
Millikin, Paul D., Proctor Community Hospital
O'Malley, John F., University of Notre Dame
Parmenter, Carol, U. S. Geological Survey
Smith, Dean O., University of Wisconsin
Valentino, Karen L., Washington University School of Medicine
Watson, Ann Y., Harvard School of Public Health
Westrum, Barbara L., Veterans Administration Medical Center

Comparative Light and Electron Microscopy in Clinical Diagnosis

Instructor-in-chief

Carter, Harry, St. Barnabas Medical Center

Other faculty, staff, and lecturers

Archuletta, Michael D., ETEC Corporation
Enders, Reinhart, E. Leitz, Inc.
Flanders, Marvin E., Carl Zeiss, Inc.
Houghton, Susan, Woods Hole Oceanographic Institution
Laudate, Anthony, JEOL-USA, Inc.
Lunn, Ron, Perkin-Elmer Corporation
Maser, Morton D, Marine Biological Laboratory
Scarborough, Ann, Louisiana State University
Solit, Elinor, Polaroid Corporation
Teplitz, Carl, Rhode Island Hospital

Students

Dominguez, Justo, Toledo Path Inc.
Gordon, Gloria, Lederle Laboratories
Husa, Carl F., Jr., Hofstra University
Kowallis, George H., St. Vincent's Hospital
Kreutzer, Donald W., Boone County Hospital
McCormick, John F., Our Lady of Lourdes Hospital
Millikin, Paul D., Proctor Community Hospital
Mutty, Danielle, V.A. Medical & Regional Office Center
O'Toole, William, Cape Cod Hospital
Wood, Edward M., Laboratory Procedures Northwest

Electron Microscopy in the Biological Sciences

Instructors-in-chief

Bowers, Blair, National Institutes of Health
Maser, Morton D, Marine Biological Laboratory

Other faculty, staff, and lecturers

Houghton, Susan, Woods Hole Oceanographic Institution
Mahon, Paul D., LKB Instruments, Inc.
Peachey, Lee D., University of Pennsylvania
Porter, Keith R., University of Colorado
Scarborough, Ann, Louisiana State University
Waterbury, John, Woods Hole Oceanographic Institution

Students

Ahmadjian, Vernon, Clark University
Blades, Pamela, Harbor Branch Foundation, Inc.
Brown, David H., Washington University School of Medicine
Brown, Rather G., Alabama A&M University
Bukovsan, William, SUNY at Oneonta
Carraway, Coralie, Oklahoma State University
Gershon, Nahum, National Institutes of Health
Hoy, Ronald R., Cornell University
Makowski, Lee, Brandeis University
Messner, Kenneth H., Milton S. Hershey Medical Center
Ortabasi, Ilse, Center for Energy & Environment Research
Squibb, Katherine, National Institute of Environmental Health Sciences

MARICULTURE: CULTURE OF MARINE INVERTEBRATES FOR RESEARCH PURPOSES

Instructor-in-chief

BERG, CARL J., JR., Harvard University/Marine Biological Laboratory

Other faculty, staff, and lecturers

BANG, FREDERIK B., Johns Hopkins University
BECK, ALLAN, Environmental Research Laboratory
BLOGOSLAWSKI, WALTER J., National Marine Fisheries Service
BRYANT, BRUCE, Boston University/Marine Biological Laboratory
CAPO, THOMAS, Marine Biological Laboratory
DOYLE, ROGER W., Dalhousie University, Canada
FARLEY, C. AUSTIN, National Marine Fisheries Service
GRASSLE, JUDITH, Marine Biological Laboratory
GUILLARD, ROBERT, Woods Hole Oceanographic Institution
HANLON, ROGER T., University of Texas, Galveston
HUGHES, JOHN T., Massachusetts State Lobster Hatchery
JANNASCH, HOLGER, Woods Hole Oceanographic Institution
LEIBOVITZ, LOUIS, Cornell University
MARCUS, NANCY H., Woods Hole Oceanographic Institution
SINDERMANN, CARL J., National Marine Fisheries Service

Students

BAGINSKI, RICHARD, University of California, Riverside
BELL, KATHRYN, McGill University, Canada
ENTENMANN, BARBARA, University of Miami
GOEHLE, KENNETH H., ECO Research Inc.
HARGREAVES, BRUCE, Lehigh University
KREJCI, MARK, University of Texas
MOUSSALLI, ELIE I., Scripps Institute of Oceanography
PHLEGER, CHARLES, San Diego State University
SICKEL, JAMES, Murray State University
SIEWERS, A. KIM, Martha's Vineyard Shellfish Group
SMAYDA, THEODORE, University of Rhode Island
STEVENS, DAVE, AMERON
TRUESDALE, FRANK, Louisiana State University
YOUNG, JEFFREY, Humboldt State University
RICE, MARY, Smithsonian Institution

LIQUID SCINTILLATION COUNTING IN BIOLOGY AND MEDICINE

Insiructor-in-chief

KOBAYASHI, YUTAKA, New England Nuclear Corporation

Other faculty, staff, and lecturers

CORLISS, THERESA, Marine Biological Laboratory
KIZUKA, HIRO, New England Nuclear Corporation
LITT, GERALD, New England Nuclear Corporation
POIRIER, DONNA, New England Nuclear Corporation

Students

BARMACK, NEAL, Good Samaritan Hospital and Medical Center
BERG, MICHAEL, New England Nuclear Corporation

Cole, Lucille, Merck, Sharp & Dohme Research Laboratories
DiStefano, John, Northport V.A. Medical Center
Gilman, Sara, Naval Submarine Medical Research Lab
Hintz, Ada Mae, National Animal Disease Center
Jackson, Eugene, Jr., University of Mississippi
Jordon, Frank, Rutgers University
King, Joe, Murray State University
Ramsden, Hugh, J. T. Baker Chemical Company
Robichaud, Caron, Yale University
Rommel, Frederick, Plum Island Animal Disease Center
Vekens, Lorraine, New England Nuclear Corp.
Weber, Leslie, Stauffer Chemical Company
Williams, Rebecca, Stauffer Chemical Company
Wright, Custer Lee, Merrell Research Center

Optimal Microscopy and Photomicrography in the Biomedical Sciences

Instructor-in-chief

Allen, Robert D., Dartmouth College

Other faculty, staff, and lecturers

Allen, Nina S., Dartmouth College
Anderson, Richard, Nikon, Inc.
Chaisson, Richard, Olympus Corp. of America
Cohen, David, Venus Scientific, Inc.
Comolla, Peri, AZI Corporation
DeCastro, Robert, AZI Corporation
Decker, Melvin, Opti-Quip, Inc.
Devine, Dana, Boston University/Marine Biological Laboratory
Dietrich, Peter, Carl Zeiss, Inc.
Gilblom, David, Hamamatsu Systems, Inc.
Goldman, Robert D., Carnegie-Mellon University
Houghton, Susan, Woods Hole Oceanographic Institution
Keller, Ernst, Carl Zeiss, Inc.
Levy, Stuart, E. Leitz, Inc.
Scheier, Kurt, Nikon, Inc.
Schneider, Richard, E. Leitz, Inc.
Scott, Martin, Eastman Kodak Company
Simon, Glenn, E. Leitz, Inc.
Spencer, Todd, Dartmouth College
Swift, Stephen, Swift Instruments, Inc.
Travis, James, Dartmouth College
Zackroff, Robert, Carnegie-Mellon University

Students

Aaron, Bev, WPVI-TV
Abbey, Michael, St. Joseph Medical Center
Barclay, Gregor, University of Waterloo, Canada
Bergmann, Katherine, Brown University
Bolz, George, National Marine Fisheries Service
Burton, Jarrett, Cryobiology Laboratory
del Cerro, Manuel, University of Rochester
Chase, Robert S., Lafayette College
Coble, Anna Jane, National Institutes of Health
Cole, Madison, Jr., Loyola University

Lum, Patrick, U. S. Department of Agriculture
Matlaga, Barbara, Ethicon Research Foundation
Nuttall, Robert P., Emory University
Persson, Bo-Eric, National Institutes of Health
Roberts, Douglas, Pacific Optical Specialists
Schneider, Kimber, Scheie Eye Institute
Walter, Robert J., Jr., University of California, Irvine

Quantitative Analysis of Electron Micrographs

Instructor-in-chief

Peachey, Lee D., University of Pennsylvania

Other faculty, staff, and lecturers

Eisenberg, Brenda, Rush Medical College
Haselgrove, John, University of Pennsylvania
Houghton, Susan, Woods Hole Oceanographic Institution
Tracey, Gregory, Marine Biological Laboratory

Students

Basgen, John, University of Minnesota Medical School
Bunick, Gerard J., Oak Ridge National Laboratory
Davidowitz, Jacob, New York University
Flor, William J., Armed Forces Radiobiology Research Institute
Hartwig, Virginia M., Armed Forces Radiobiology Research Institute
Jones, Betty Ruth, Morehouse College
Kirby, Gerald, Tulane Medical Center
Laughlin, Tommie J., Oak Ridge National Laboratory
Makuchan, Eileen M., University of Hawaii
Molsen, Dian, Northern Illinois University
Murphy, William, Naval Regional Medical Center
Osage, Justine E., Beth Israel Hospital
Paul, Rex N., Jr., U. S. Department of Agriculture
Pham, Tuan Duc, Columbia University
Poole, Max C., East Carolina University
Rosenfield–Wessells, Sheila, Eastern Virginia Medical School
Scott, Grayson, University of Wisconsin
Sternlicht, Himan, Case Western Reserve University
Stovall, Johnnie Belinda, NORDA

X. RESEARCH AND TRAINING PROGRAMS

SUMMER

Principal Investigators

Adams, James, Tennessee State University
Armstrong, Clay M., University of Pennsylvania
Armstrong, Peter B., University of California, Davis
Arnold, John M., University of Hawaii, Kewalo Marine Laboratory
Atwood, Harold L., University of Toronto, Canada
Ball, Eric G., Harvard Medical School
Barlow, Robert B., Jr., Syracuse University

Bartlett, Grant R., Laboratory for Comparative Biochemistry
Bauer, G. Eric, University of Minnesota
Beauge, Luis A., University of Maryland School of Medicine
Bell, Eugene, Massachusetts Institute of Technology
Bennett, Michael V. L., Albert Einstein College of Medicine
Bishop, Stephen H., Iowa State University
Borgese, Thomas A., CUNY, Lehman College
Bourne, George B., University of Calgary, Canada
Brodwick, Malcolm S., University of Texas Medical Branch
Brown, Joel E., SUNY at Stony Brook
Burdick, Carolyn J., Brooklyn College
Burger, Max M., University of Basel, Switzerland
Catapane, Edward J., CUNY, Merger Evers College
Chang, Donald C., Baylor College of Medicine
Chappell, Richard L., Hunter College
Charlton, Milton P., University of Toronto, Canada
Cohen, Adolph I., Washington University School of Medicine
Cohen, Lawrence B., Yale University School of Medicine
Cohen, William D., Hunter College
Cooperstein, Sherwin J., University of Connecticut School of Medicine
deTerra, Noel, Hahnemann Medical College
DeWeer, Paul, Washington University School of Medicine
DuBois, Arthur B., John B. Pierce Foundation Laboratory
Eaton, Douglas C., University of Texas Medical Branch
Eckberg, William R., Howard University
Farmanfarmaian, A., Rutgers University
Fishman, Harvey M., University of Texas Medical Branch
Gainer, Harold, National Institutes of Health
Geduldig, Donald, Memorial University of Newfoundland, Canada
Gilbert, Daniel L., National Institutes of Health
Goldman, Robert D., Carnegie-Mellon University
Goldsmith, Timothy, Yale University
Goode, Dennis, University of Maryland
Gray, Paulette S., Atlanta University
Grosch, Daniel S., North Carolina State University
Gross, Cordell E., University of Iowa
Grossfeld, Robert M., University of Texas
Grossman, Albert, New York University Medical Center
Harding, Clifford V., Wayne State University School of Medicine
Haschemeyer, Audrey E. V., Hunter College
Hepler, Peter K., University of Massachusetts
Highstein, Stephen M., Albert Einstein College of Medicine
Hogan, James C., Jr., University of Connecticut
Hoffmann, Richard J., Iowa State University
Hoskin, Francis C. G., Illinois Institute of Technology
Hufnagel, Linda, University of Rhode Island
Humphreys, Susie H., National Institute on Aging
Ilan, Joseph, Case Western Reserve University
Jacobs-Lorena, Marcelo, Case Western Reserve University
Jordan, Thomas L., Dillard University
Josephson, Robert K., University of California, Irvine
Joyner, Ronald W., University of Iowa
Kao, C. Y., SUNY, Downstate Medical Center
Koide, Samuel S., Population Council, Rockefeller University
Kuffler, Stephen W., Harvard Medical School
Kusano, Kiyoshi, Illinois Institute of Technology

Landowne, David, University of Miami
Langford, George, Howard University
Lasek, Raymond J., Case Western Reserve University
Lash, James W., University of Pennsylvania
Laufer, Hans, University of Connecticut
Lee, John J., City College of CUNY
Lerman, Sidney, Emory University
Lester, Henry H., California Institute of Technology
Levitan, Irwin B., Friedrich Miescher Institut
Lipicky, Raymond, University of Cincinnati
Llinas, Rodolfo, New York University Medical Center
Lockwood, Arthur, University of North Carolina
Loewenstein, Werner R., University of Miami School of Medicine
Maglott, Donna R., Howard University
Mastroianni, Luigi, Jr., University of Pennsylvania
Metuzals, Janis, University of Ottawa, Canada
Metz, Charles B., University of Miami
Mitchell, Ralph, Harvard University
Moore, John W., Duke University Medical Center
Moore, Lee E., University of Texas Medical Branch
Mullins, L. J., University of Maryland School of Medicine
Nagel, Ronald L., Albert Einstein College of Medicine
Narahashi, Toshio, Northwestern University Medical School
Nicosia, Santo V., University of Pennsylvania School of Medicine
Noe, Bryan D., Emory University
Oxford, Gerry S., University of North Carolina
Pappas, George D., University of Illinois, College of Medicine
Person, Philip, Veterans Administration Medical Center
Pierce, Sidney K., University of Maryland
Pollard, Harvey B., National Institutes of Health
Przybylski, Ronald J., Case Western Reserve University
Ramon, Fidel, Duke University Medical Center
Rebhun, Lionel J., University of Virginia
Reese, Thomas S., National Institutes of Health
Reynolds, George T., Princeton University
Rickles, Frederick R., University of Connecticut Health Center
Ripps, Harris, New York University School of Medicine
Rushforth, Norman B., Case Western Reserve University
Russell, John M., University of Texas Medical Branch
Russell-Hunter, W. D., Syracuse University
Rustad, Ronald C., Case Western Reserve University
Sachs, John R., SUNY at Stony Brook
Saffo, Mary B., Swarthmore College
Salzberg, Brian M., University of Pennsylvania School of Dental Medicine
Sanger, Joseph W., University of Pennsylvania School of Medicine
Schuel, Herbert, SUNY at Buffalo
Schuetz, Allen W., Johns Hopkins University
Schwab, Walter E., Virginia Polytechnical Institute and State University
Shrivastav, Brij B., Duke University Medical Center
Silverberg, Sidonie A., SUNY at Stony Brook
Speck, William T., Case Western Reserve University
Spiegel, Melvin, Dartmouth College
Starzak, Michael, SUNY at Binghampton
Stetten, Marjorie R., National Institutes of Health
Stokes, Darrell R., Emory University
Stuart, Ann E., Harvard Medical School

Stunkard, Horace W., American Museum of Natural History
Szamier, R. Bruce, Massachusetts Eye and Ear Infirmary
Szent-Gyorgyi, Andrew, Brandeis University
Tasaki, Ichiji, National Institutes of Health
Treistman, Steven N., Bryn Mawr College
Trinkaus, John P., Yale University
Troll, Walter, New York University Medical Center
Troxler, Robert F., Boston University
Walker, Dorothy G., Howard University
Wallace, Robin A., Oak Ridge National Laboratories
Weidner, Earl, Louisiana State University
Weissmann, Gerald, New York University Medical Center
West, David L., Sangamon State University
Whittaker, J. Richard, Wistar Institute
Wolf, Don, University of Pennsylvania School of Medicine
Zigman, Seymour, University of Rochester Medical Center

Library Readers

Adelberg, Edward, Yale University
Allen, Garland E., Washington University
Allen, Nina Stromgren, Dartmouth College
Allen, Robert D., Dartmouth College
Atkinson, Burr G., University of Western Ontario, Canada
Baltimore, David, Massachusetts Institute of Technology
Barbieri, Francisco, Universidad Nacional de Tucuman, Argentina
Bean, Charles, General Electric Company
Bechtol, Kathleen, Wistar Institute
Beidler, L. M., Florida State University
Bolton, James R., University of Western Ontario, Canada
Brown, Frank A., Jr., Marine Biological Laboratories
Bryant, Susan, University of California, Irvine
Carriere, Rita M., SUNY, Downstate Medical Center
Chambers, Edward, University of Miami, School of Medicine
Cohen, Maynard M., Rush Medical College
Cole, Kenneth S., National Institutes of Health
Dettbarn, Wolf D., Vanderbilt University, School of Medicine
Diamond, Jack, McMaster Medical Centre
Dodge, Frederick A., The Rockefeller University
Ebert, James D., Carnegie Institution of Washington
Dientsman, Steven, New York University, School of Medicine
Edds, Louise L., Ohio University
Eder, Howard A., Albert Einstein College of Medicine
Edward, C. (Brother), Manhattan College
Fischman, Donald A., State University of New York, Downstate Medical Center
Fisher, Saul H., New York University School of Medicine
Franzini-Armstrong, Clara, University of Pennsylvania
Fussell, Catherine P., Pennsylvania State University
Gabriel, Mordecai L., Brooklyn College
Galatzer-Levy, Robert M., University of Chicago
Goldberg, Alfred L., Harvard Medical School
Goldstein, Moise H., Jr., The Johns Hopkins University
Goudsmit, Esther M., Oakland University
Grant, Philip, University of Oregon
Guttman, Rita, CUNY, Brooklyn College
Harrington, Cynthia, University of Wisconsin

Hinsch, Gertrude W., University of South Florida
Huebner, Erwin, University of Manitoba
Huettner, Robert J., Columbia University, School of Dentistry
Isenberg, Irvin, Oregon State University
Jeffery, William R., University of Texas, Austin
Kaminer, Benjamin, Boston University, School of Medicine
Karush, Fred, University of Pennsylvania, School of Medicine
Kilham, Peter, University of Michigan
Kirschenbaum, D., SUNY, College of Medicine
Kosower, Edward M., Tel-Aviv University
Kogut, Margot, University of London, King's College, England
Krupa, Paul, CUNY, City College
Laderman, Aimlee, SUNY, Binghamton
Lazarow, Jane K., Minnesota Department of Health
Lazarow, Paul B., The Rockefeller University
Leighton, Joseph, Medical College of Pennsylvania
Levitz, Mortimer, New York University Medical Center
Lorand, Laszlo, Northwestern University
Mizell, Merle, Tulane University
Morrell, Frank, Rush Medical College
Niederman, Richard, Veterans Administration Medical Center
Norris, William E., Jr., Southwest Texas State University
Ojakian, George, SUNY Downstate Medical Center
Pappas, George, University of Illinois, College of Medicine
Pearlman, Alan L., Washington University School of Medicine
Pederson, Judith B., Holy Cross College
Pederson, Thoru, Worcester Foundation for Experimental Biology
Plocke, Donald J. S. J., Boston College
Porter, Keith, University of Colorado
Prosen, Edward J., National Bureau of Standards
Prosser, C. Ladd, University of Illinois
Quigley, James P., SUNY, Downstate Medical Center
Reiner, John M., Albany Medical College of Union University
Rice, Robert V., Carnegie-Mellon Institute
Rohr, Kristin M., Woods Hole Oceanographic Institution
Rosenbaum, Joel L., Yale University
Roth, Jay S., University of Connecticut
Rowland, Lewis P., College of Physicians and Surgeons
Rubinow, Sol I., Cornell University Medical Center
Salmon, Edward D., University of North Carolina
Schlesinger, R. Walter, Rutgers Medical School, College of Medicine and Dentistry of New Jersey
Scott, George T., Oberlin College
Selman, Kelly, College of Medicine, University of Florida
Shemin, David, Northwestern University
Shepro, David, Boston University
Sherman, Irwin W., University of California
Sonnenblick, B. P., Rutgers University
Spector, Abraham, Columbia University
Strittmatter, Cornelius F., Bowman Gray School of Medicine
Taber, Robert, State of New York, Department of Health
Tweedell, Kenyon S., University of Notre Dame
Wainio, Walter, Rutgers College
Waksman, Byron H., Yale University School of Medicine
Warren, Leonard, Wistar Institute
Webb, H. Marguerite, Goucher College

Wheeler, George E., Brooklyn College
Wilber, Charles G., Colorado State University
Wilson, Thomas H., Harvard Medical School
Wittenberg, Jonathan B., Albert Einstein College of Medicine
Yntema, Chester L., SUNY, Upstate Medical Center
Zacks, Sumner I., The Miriam Hospital
Zusy, Dennis R., Clarke College

Other Research Personnel

Adejuwon, Christopher, Rockefeller University
Akabas, Myles, Albert Einstein College of Medicine
Anderson, Paul J., New York University School of Medicine
Anderson, Peter A. V., University of St. Andrews, Scotland
Antonellis, Blenda, Case Western Reserve University
Arnold, Lois D. W., University of Hawaii, Kewalo Marine Laboratory
Aljure, E., Albert Einstein College of Medicine
Bagnell, Carol A., Medical College of Georgia
Bahner, Joan A., Tennessee State University
Baron, Jeffrey, Rice University
Bartelt, Diana, Hunter College, CUNY
Bath, Donna, University of Ottawa, Canada
Baumgold, Jesse, National Institutes of Health
Behrman, Michael, Williams College
Best, Joanne E., University of Connecticut
Bito, Laszlo Z., Columbia University
Bloom, George, University of North Carolina
Boron, Walter F., Yale University School of Medicine
Bosler, Robert B., Harvard Medical School
Boyle, Mary, Yale University School of Medicine
Breitwieser, Gerda E., Washington University Medical School
Brown, Stanley B., University of Leeds, England
Buck, John, National Institutes of Health
Burkart, Werner, University of Basel, Switzerland
Cappell, Mitchell S., Albert Einstein College of Medicine
Chilton, Beverly, University of Pennsylvania Medical School
Clapin, David, University of Ottawa, Canada
Clark, John, Michigan State University
Coburn, Michael, New York University Medical Center
Cohen, Richard, Hunter College, CUNY
Corey, Jody P., University of Basel, Switzerland
Couch, Ernest, Texas Christian University
Croop, Robert, University of Pennsylvania
Cummins, Dean R., Yale University
Czekanski, Sandra, University of Alberta, Canada
Dahl, Robert F., Princeton University
DiPolo, Reinaldo, Instituto Venezolano de Investigaciones Cientificas, Venezuela
Drake, Peter F., Bryn Mawr College
Dudziak, Denise, University of North Carolina
Dunham, Philip, Syracuse University
Eckert, Richard C., CUNY, Herbert Lehman College
Edwards, Samuel C., University of Maryland
Eng, Douglas, Amherst College
Eng, Ludeman, University of Miami
Farley, Jerry, Northwestern University Medical School
Fish, R. David, University of Texas Southwestern Medical School
Flanagan, Tracy, Case Western Reserve University

Folman, Rachel, The Hebrew University, Israel
Fuseler, John W., University of Texas
Gehrke, Lee, Case Western Reserve University
Gibbons, Robert C., National Institutes of Health
Gilly, William F., University of Pennsylvania
Goldman, Anne E., Carnegie-Mellon Institute
Goldsmith, Paul K., National Institutes of Health
Goodman, Elizabeth, New York University Medical School
Goodman, Lesley L., Queen Mary College, University of London, England
Gordon, Valery, University of Pennsylvania
Grant, Neva, University of California, San Francisco
Greenwalt, Dale, Iowa State University
Grupp, Stephan, University of Cincinnati
Guy, Richard Grenville, City University of London, England
Halm, Dan, University of Iowa
Hanna, Robert B., SUNY at Syracuse
Harris, Andrew L., Albert Einstein College of Medicine
Harris, Edward M., Duke University Medical Center
Hellman, Geoffrey, Indiana University
Hines, Michael, Duke University
Hochberg, Abraham, The Hebrew University, Israel
Holderbaum, Roxanna, Rockefeller University
Howard, Louisa, Dartmouth College
Huse, William, Albert Einstein School of Medicine
Hutchison, Nancy, University of California, Irvine
Ii, Ichio, University of Virginia
Inhof, Ruth, University of Basel, Switzerland
Inoue, Sadayuki, McGill University, Canada
Isaacson, J. Harry, Wayne State University
Iwasa, K., National Institutes of Health
Jackson, Andrea M., Howard University
Jacobsen, Freda S., University of Cincinnati
James, Marilyn E., Tennessee State University
Jeffrey, Chris, Emory University
Jimenez, Ramon N., New York City Medical Center
Johnson, Judith, Medgar Evers College, CUNY
Josephson, Elizabeth A., Wistar Institute
Judge, Susan, University of Southampton, England
Kaplan, Ehud, Rockefeller University
Kao, Peter, SUNY, Downstate Medical Center
Keller, Thomas, University of Virginia
Kelly, Susan J., Case Western Reserve University
Kirchman, David, Harvard University
Kirk, Kevin L., University of Iowa
Kirsch, Glenn E., Northwestern University
Ko, Chien-Ping, National Institutes of Health
Kracke, George R., Washington University School of Medicine
Kremer, Norbert E., Purdue University
Kriebel, Mahlon, University of Illinois College of Medicine
Krouse, Mauri E., California Institute of Technology
Laufer, Marc R., University of Pennsylvania
Lee, Monica J., Bucknell University
Lesher, Sarah, Yale University
Lettieri, Jerry, Cornell University
Lipman, Debbe, University of Rochester
Lippincott, Frances B., Cornell University

Lo, Woo-Kuen, Wayne State University
Lund, Albert E., Northwestern University Medical School
Lyn-Cook, Lascelles, Howard University
Marcano, Juan Armando, Medgar Evers College, CUNY
Maruo, Takeshi, Rockefeller University
Mason, Vincent R., Howard University
Mathews, Rita W., Hunter College
Matsumura, Fumio, Michigan State University
Matteson, Donald R., University of Pennsylvania
Mazanet, Rosemary, University of Pennsylvania
McKinney, Leslie C., Washington University School of Medicine
Meedel, Thomas H., Wistar Institute
Megaw, Judith M., Emory University
Mercuro, T. John, Rutgers University
Meshul, Charles K., University of Illinois Medical Center
Mir-Lechaire, Francoise, University of Basel, Switzerland
Misevic, Gradimir, University of Basel, Switzerland
Mobbs, Peter G., Centre for Overseas Pest Research, England
Momii, Akira, Rockefeller University
Moran, Michael N., Emory University
Morris, James R., Case Western Reserve University
Nagaswami, Chandrasekaran, University of Pennsylvania School of Medicine
Nakatsuji, Norio, Massachusetts Institute of Technology
Nakatsuji, Takako, Massachusetts Institute of Technology
Naranjo, Ramon, New York University Medical Center
Nemhauser, Irsi, Hunter College, CUNY
Obaid, Ana Lia, University of Pennsylvania Medical School
Oertel, Donata, University of Wisconsin
Ohki, Shinpei, SUNY at Buffalo
Ornberg, Richard L., National Institutes of Health
Ovadia, Michael, University of Pennsylvania
Parker, Charles H., University of Pennsylvania
Parmentier, James, Duke University Medical Center
Parnas, Itzhak, The Hebrew University, Israel
Paxhia, Teresa M., University of Rochester
Persell, Roger, Mercy College
Poussart, Denis, University of Loval, Canada
Poznansky, Mark, University of Alberta, Canada
Prusch, Robert D., Rhode Island College
Przybylski, Michelle, Case Western Reserve University
Quandt, Fred N., Northwestern University
Quinn, Christopher, Syracuse University
Rapport, Seth L., University of Connecticut Health Center
Requena, Jaime, Instituto Venezolano de Investigaciones Cientificas, Venezuela
Rebhun, Linda, University of Hawaii
Robbins, Daniel S., Howard University
Robertson, Lola E., American Museum of Natural History
Rose, Birgit, University of Miami, School of Medicine
Rosenkranz, Pnina, Barnard College
Rudensey, Lyle M., Case Western Reserve University
Rushforth, Nancy M., Case Western Reserve University
Sanger, Jean M., University of Pennsylvania School of Medicine
Scarborough, Ann, Louisiana State University
Schessel, David, Albert Einstein College of Medicine
Schmeidler, Katherine T., Case Western Reserve University
Schnapp, Bruce, Harvard Medical School

Scruggs, Virginia, Northwestern University Medical School
Segal, Sheldon J., Rockefeller Foundation
Sejnowski, Terrence J., Harvard Medical School
Selick, Harold E., University of Pennsylvania
Selman, Kelly, University of Florida College of Medicine
Serhan, Charles N., New York University Medical Center
Ship, Jonathan, University of Pennsylvania
Shoup, Thomas Lee, Emory University
Shure, Michael, Yale University
Siegel, Irwin, New York University Medical Center
Simon, Sanford, New York University Medical School
Simon, Sidney A., Duke University
Simpson, T., University of Basel, Switzerland
Sims, Steve, Columbia University
Skell, Jeffrey, University of Texas Medical Branch
Smith, H. Gilbert, Jr., Washington University
Socci, Robin R., Rutgers University
Solomon, Dennis J., Massachusetts Institute of Technology
Spiegel, Evelyn, Dartmouth College
Spira, Micha E., Albert Einstein College of Medicine
Spray, David C., Albert Einstein College of Medicine
Stathoplos, Linda, Swarthmore College
Steele, Joy A., University of Alberta, Canada
Steinacher, Antoinette, Rockefeller University
Stern, Vera, Case Western Reserve University
Stracher, Alfred, New York University Medical Center
Sugimori, Mutsuyuki, New York University Medical Center
Susan, Stanley R., Wayne State University
Susswein, Abraham J., Albert Einstein College of Medicine
Svendsen, Clive Niels, Slade House Farm, England
Swenson, Randolphe P., University of Pennsylvania
Szentkiralyi-Szent-Gyorgyi, Eva, Brandeis University
Takei, Yoshio, Emory University School of Medicine
Tauck, David L., Duke University
Terakawa, Susumu, National Institutes of Health
Thompson, Charlie S., University of Toronto, Canada
Timpe, Leslie C., Harvard Medical School
Tirosh, Reuven, National Institutes of Health
Tormey, John, University of California, Los Angeles
Tytell, Michael, Case Western Reserve University
Viles, J. M., Iowa State University
Wagner, Eric S., Cook College, Rutgers University
Wagner, Richard W., University of Miami School of Medicine
Wahl, Michael, Case Western Reserve University
Waldron, John, National Institutes of Health
Wang, Howard H., University of California, Santa Cruz
Warren, Mary K., University of Maryland
Watanabe, Akira, Tokyo Medical and Dental University, Japan
Weber, Annemarie, University of Pennsylvania
Weibel, Timothy, Duke University Medical Center
Weinstock, Martin, California Institute of Technology
Westerfield, Monte, Harvard Medical School
Wick, Robert, Case Western Reserve University
Williams, Carolyn H., Emory University
Wilson, Darcy B., University of Pennsylvania School of Medicine
Winters, Thomas, University of Connecticut

Wolniak, Stephen M., University of Massachusetts
Worgul, Basil, Columbia University
Wu, Chau H., Northwestern University Medical School
Yeh, Jay Z., Northwestern University Medical School
Yulo, Teresa, University of Rochester Medical Center
Zackroff, Robert V., Carnegie-Mellon University
Zakevicius, Jane M., New York University School of Medicine
Zimering, Mark B., Albert Einstein College of Medicine

YEAR-ROUND PROGRAMS

Boston University Marine Program (BUMP)

Director

Humes, Arthur G., Professor of Biology, Boston University

Staff

Allen, Sarah, Boston University
Atema, Jelle, Boston University
Beale, Eleanore, Boston University
Butler, Norma, Boston University
Cogswell, Charlotte, Boston University
Elgin, Randall, Boston University
Gibbons, Shelley, Boston University
Govind, C. K., University of Toronto, Canada
Hahn, Dorothy, Boston University
Harrington, Robin, Boston University
Hill, Lena, Boston University
Karnofsky, Lisa, Boston University
Lewandowski, Ann, Boston University
Meiss, Dennis, Clark University
Oleszko-Szuts, Susan, Boston University
Pearce, Jeanne, University of Toronto, Canada
Price, Christopher, Boston University
Rietsma, Carol, SUNY at New Paltz
Tamm, Sidney L., Boston University
Tamm, Singhild
Valiela, Ivan, Boston University
Van Etten, Richard, Woods Hole Oceanographic Institution
Wolkmann, Suzanne, Woods Hole Oceanographic Institution

Trainees (all of Boston University)

Aaronson, Rebecca
Ashendorf, Deborah
Ashkenas, Linda
Botero, Leonor
Bryant, Bruce
Buchsbaum, Robert
Caraco, Nina
Cole, James
Cole, Timothy
Cushman, Mary
Davis, Cabell
Derby, Charles
Devine, Dana
Dojiri, Masahiro
Dourdeville, Theodore
Duncan, Thomas
Foreman, Kenneth
French, Kathleen
Frommel, Thomas
Giblin, Anne
Gibson, Daniel
Hill, Russell
Hook, Jamie
Howes, Bryan

Joe, Adam
Jordan, Thomas
Kent, Karla
Kilpatrick, Katherine
Kolba, Clifford
Maciolek, Nancy
Mareb, Frederick
Martin, Michael
Mautino, Michele
Pascoe, Natalie
Poole, Alan
Smith, Polly
Swanson, Laureen
Trott, Thomas
Walthall, Walter
Webb, Jacqueline
Werme, Christine
Wier, Susan
Williams, Isabelle
Wilson, John
Yerger, George

The Ecosystems Center

Director

George M. Woodwell, Marine Biological Laboratory

Staff (all of Marine Biological Laboratory)

Ammons, J. Timothy
Behrens, William
Boone, Richard D.
Bowles, Francis P.
Burroughs, Richard H.
Campbell, Nancy
Carlson, Chris
Cavanaugh, Colleen
Clark, Joanne
Corliss, Teresa A. L.
Cvikevich, Anya
Eldred, Kate
Elkin, Kerry M.
Eshleman, Keith N.
Fay, Jamie
Fownes, James N.
Friedlander, Amy I.
Gale, Judith A.
Helfrich, John
Hobbie, John E.
Houghton, Richard A.
Howarth, Robert W.
Juers, David W.
Kane, Ann E.
Kane, Mary
Kijowski, Voytek
Lajtha, Kathryn A.
Lanyon, Cynthia
Lipschultz, Frederic
Luchessa, Karen
Meisner, Benedicte
Melillo, Jerry M.
Mlodniska, Kasia
Montgomery, Mary Louise
Morse, Julie K.
Morris, James T.
Nilson, Carol
Palm, Cheryl A.
Parsons, Katherine C.
Peterson, Bruce J.
Scheltema, Konrad
Schimel, David
Schimel, Joshua
Semino, Suzanne
Shaver, Gaius R.
Shelkey, Jeffrey B.
Sladovich, Hedy E.
Steudler, Paul A.
Turner, Andrea R.
Upton, Joan M.
White, Mark
Zacks, Charles

Trainees

Bowden, W. Breck, University of North Carolina, Year-in-Science
Keene, Janet, Yale University, Year-in-Science Summer Intern
Klinger, Alan, Yale University, Year-in-Science Summer Intern
McClaugherty, Charles M., University of Virginia, Year-in-Science
Reed, James P., University of North Carolina, Year-in-Science
Shen, Susan, Yale University, Year-in-Science Summer Intern

DEVELOPMENTAL AND REPRODUCTIVE BIOLOGY LABORATORY

Director

GROSS, PAUL R., Marine Biological Laboratory

LABORATORY OF BIOPHYSICS

Director

ADELMAN, WILLIAM J., JR., NINCDS-NIH

Staff

ALKON, DANIEL L., NINCDS-NIH
BELAMARICH, PETER F., Boston University Medical School
BUCHANAN, JOANN, NINCDS-NIH
DEFELICE, Louis, Emory University
FOHLMEISTER, JURGEN F., University of Minnesota
FRENCH, ROBERT J., NINCDS-NIH
GOLDMAN, DAVID E., SUNY at Binghamton
HALTIWANGER, ROBERT S., Duke University
HARRIGAN, JUNE F., NINCDS-NIH
HODGE, ALAN J., NINCDS-NIH
JERUSSI, THOMAS P., NINCDS-NIH
KUZIRIAN, ALAN, NINCDS-NIH
KUZIRIAN, JEANNE, NINCDS-NIH
LEDERHENDLER, I. IZJA, NINCDS-NIH
LEONARD, DOROTHY A., NINDCS-NIH
MANNING, MICHAEL, NINCDS-NIH
MORRISON, PAUL T., NINCDS-NIH
MUELLER, RUTHANNE, NINCDS-NIH
NEARY, JOSEPH T., NINCDS-NIH
OLDS, JAMES, University of California, Irvine
SENFT, STEPHEN L., University of Oregon
SHIMAN, LEON G., NINCDS-NIH
SHOUKIMAS, JONATHAN J., NINCDS-NIH
STEELE, HUGH, NINCDS-NIH
TABATA, MITSUO, NINCDS-NIH
TAKEDA, TOSHIAKI, NINCDS-NIH
TYNDALE, CLYDE, NINCDS-NIH
WALTZ, RICHARD, NINCDS-NIH
WELLS, JAY B., NINCDS-NIH
WEST, ALEXANDER, NINCDS-NIH
WHITING, LIANE E., NINCDS-NIH
WIERCINSKI, FLOYD, Northeastern Illinois University

LABORATORY OF SENSORY PHYSIOLOGY

Director

MACNICHOL, EDWARD F., JR., Marine Biological Laboratory

Staff

CAMPBELL, NANCY, Marine Biological Laboratory
COLLINS, BARBARA ANN, Marine Biological Laboratory
CORSON, D. WESLEY, Marine Biological Laboratory

Fein, Alan, Marine Biological Laboratory
French, E. Kathleen, Marine Biological Laboratory
Goodman, Steven, Marine Biological Laboratory
Harosi, Ferenc I., Marine Biological Laboratory
Levine, Joseph S., Marine Biological Laboratory
Szuts, Ete Zoltan, Marine Biological Laboratory
Villee, Stephen, Marine Biological Laboratory
Williams, Philip, Marine Biological Laboratory

National Foundation for Cancer Research

Director

Szent-Gyorgyi, Albert, Marine Biological Laboratory

Staff

Bone, Stephen, Marine Biological Laboratory
Gascoyne, Peter R. C., Marine Biological Laboratory
Lewis, T. John, University College of North Wales, United Kingdom
McLaughlin, Jane A., Marine Biological Laboratory
Meany, Richard A., Marine Biological Laboratory
Pethig, Ronald, University College of North Wales, United Kingdom
Selig, Joshua, Marine Biological Laboratory

Laboratory of D. Eugene Copeland

Director

Copeland, D. Eugene, Marine Biological Laboratory

Laboratory of Kenneth Edds

Director

Edds, Kenneth, Marine Biological Laboratory

Staff

Jarvis, Norman, Marine Biological Laboratory

Laboratory of Judith P. Grassle

Director

Grassle, Judith P., Marine Biological Laboratory

Staff

Birtwistle, Fern, Marine Biological Laboratory
Philbin-Munson, Maybelline, Marine Biological Laboratory
Smith, Jennifer, Marine Biological Laboratory

Laboratory of Shinya Inoué

Director

Inoué, Shinya, University of Pennsylvania/Marine Biological Laboratory

Staff

Eisen, Andrew, University of Pennsylvania
Feinberg, Mark, University of Pennsylvania

Laboratory of Eric Kandel

Director

Kandel, Eric, Columbia University

Staff

Berg, Carl J., Jr., Columbia University/Harvard University
Capo, Thomas, Columbia University
Paige, John, Columbia University/Cornell University
Perritt, Susan, Columbia University
Rubinow, Jerry, Marine Biological Laboratory

Laboratory of Keith R. Porter

Director

Porter, Keith R., University of Colorado

Staff

Beckerle, Mary C., University of Colorado
Byers, H. Randolph, Harvard Medical School
Goncalves, Mark A., University of Colorado
McNiven, Mark, Marine Biological Laboratory

Laboratory of Robert V. Rice

Director

Rice, Robert V., Carnegie-Mellon University

Staff

Houghton, Susan, Carnegie-Mellon University
Lash, Rebecca, Carnegie-Mellon University
Roslansky, Priscilla, Carnegie-Mellon University

Laboratory of Raymond E. Stephe

Director

Stephens, Raymond E., Marine Biological Laboratory

Staff

Otter, Timothy, University of North Carolina
Porter, Mary E., University of Pennsylvania
Pratt, Melanie, Marine Biological Laboratory
Stommel, Elijah, Marine Biological Laboratory
Suprenant, Kathy, University of Virginia

LABORATORY OF LEWIS G. TILNEY

Director

TILNEY, LEWIS G., University of Pennsylvania

Staff

JAFFE, LAURINDA A., University of Pennsylvania

LABORATORY OF RUTH D. TURNER

Director

TURNER, RUTH D., Harvard University

Staff

BERG, CARL J., JR., Columbia University/Harvard University
TRACEY, GREGORY A., Harvard University

XI. HONORS

FRIDAY EVENING LECTURES

VINCENT DETHIER, University of Massachusetts, January 5
"*Chemical Ecology, Insect Feeding*"
ROBERT CAPRANICA, Cornell University, January 12
"*Frog Communication*"
KATY PAYNE, Woods Hole, Massachusetts January 19
"*The Changing Songs of Humpback Whales*"
EDWARD O. WILSON, Harvard University, June 26
"*The Organization of Insect Societies*"
RICHARD O. HYNES, Massachusetts Institute of Technology, July 6
The Edgar Zwilling Lecture, "*The Role of Surface Protein in Cell Behavior*"
STEPHEN W. KUFFLER, Harvard University, July 12, 13
Forbes Lectures, *1.* "*Different Modes of Synaptic Signaling*". *2.* "*Slow Synaptic Events and the Search for a New Transmitter*"
EDWIN H. SOUTHERN, University of Edinburgh, Scotland, U. K., July 20
"*DNA and Evolution*"
HAROLD L. ATWOOD, University of Toronto, July 27
Fred Lang Memorial Lecture, "*Claw Control in Crustaceans*"
IAN CHESTER-JONES, University of Sheffield, England, U. K., August 3
"*Hormonal Influences on Ionic Balance in Selected Vertebrates*"
LEWIS THOMAS, Sloan-Kettering Memorial Institute, August 10
"*The Sense of Self in Biology*"
STANLEY COHEN, Stanford University, August 24
"*Gene Manipulation in Microorganisms*"

GRASS FOUNDATION FELLOWS

AUGUSTINE, GEORGE, University of Maryland
BENSON, JACK A., Laboratory of Sensory Sciences
CARBONETTO, SALVATORE, Carnegie Institute of Washington
DUBINSKY, JANET, University of North Carolina
FRASER, SCOTT E., Johns Hopkins University
GOLD, GEOFFREY H., University of California at San Francisco

IADAROLA, MICHAEL J., Georgetown University
MARGIOTTA, JOSEPH F., SUNY at Stony Brook
MASUKAWA, LEONA M., Armed Forces Radiobiology Research Institute
POLLACK, GERALD S., Cornell University
REUBEN, JOHN, Columbia University, co-director
REINGOLD STEPHEN C., Princeton University
WATSON, WINSOR, III, University of New Hampshire, co-director

JOSIAH MACY, JR., FOUNDATION FELLOWS AND SCHOLARS

ADAMS, JAMES A., Tennessee State University
BROWN, RATHER G., Alabama A&M University
DAWKINS, BEVERLY ANN, Atlanta University
GRAY, PAULETTE S., Atlanta University
HICKS, HERALINE E., Atlanta University
HOGAN, JAMES C., JR., University of Connecticut
JONES, BETTY RUTH, Morehouse College
JORDAN, THOMAS L., Dillard University
SHELTON, MARK R., Colorado State University
WALKER, DOROTHY G., Howard University

STEPS TOWARD INDEPENDENCE FELLOWS

BOURNE, GEORGE B., University of Calgary, Canada
CATAPANE, EDWARD J., CUNY, Medgar Evers College
FARLEY, JOSEPH, Princeton University
HAYHOME, BARBARA, University of Nebraska
HUFNAGEL, LINDA, University of Rhode Island
KUSERK, FRANK T., Moravian College
LOCKWOOD, ARTHUR H., Univeristy of North Carolina
MAGLOTT, DONNA R., Howard University
SAFFO, MARY BETH, Swarthmore College
TREISTMAN, STEVEN N., Bryn Mawr College
WEST, DAVID L., Sangamon State University

LILLIE FELLOW

SOUTHERN, E. M., University of Edinburgh, Scottland, U.K.

SOCIETY OF GENERAL PHYSIOLOGISTS SCHOLARS

HOROVITCH, SHARON, Massachusetts Unstitute of Technology
HOWARD, ELIZABETH ANN, University of California, Berkeley
VIERLING, ELIZABETH, University of Chicago

XII. INSTITUTIONS REPRESENTED

U. S. A.

Abilene Christian University
Alabama A&M University
Albert Einstein College of Medicine
American Museum of Natural History
AMERON
Ames Laboratory, Moffett Field
Amherst College
Antioch, New England
Arizona, University of
Arizona State University
Armed Forces Radiobiology Research Institution
Assumption College
Atlanta University
AZI Corporation

J. T. Baker Chemical Co.
Barnard College
Battelle Memorial Institute
Baylor College of Medicine
Benedictine College
Bennington College
Beth Israel Hospital
Bigelow Laboratory for Ocean Sciences
Birmingham Southern College
Boone County Hospital
Boston Cancer Institute
Boston College
Boston University
Boston University Marine Program
Boston University School of Medicine
Bowman Gray School of Medicine
Brandeis University
Bridgeport, University of
Brookhaven National Laboratory
Brown University
Bryn Mawr College
Bucknell University

California Institute of Technology
California, University of, Berkeley
California, University of, Davis
California, University of, Irvine
California, University of, Los Angeles
California, University of, Medical School
California, University of, Riverside
California, University of, San Diego
California, University of, Santa Barbara
California, University of, Santa Cruz
California State University, Long Beach
Cancer Research Institute
Cape Cod Hospital
Cape Cod Planning & Economic Development Commission
Carl Zeiss Inc.
Carnegie Institution of Washington
Carnegie-Mellon University
Case Western Reserve University
Center for Energy & Environmental Research
Chicago, University of
Chicago Medical School
Cincinnati, University of
Clark College
Clark University
Clemson University
Colorado, University of
Colorado, University of, Medical School
Colorado State University
Columbia University
Columbia University, College of Physicians & Surgeons
Columbia University, School of Dentistry
Connecticut, University of
Connecticut, University of, Health Center
Connecticut, University of, Medical School
Cook College
Cornell Medical College
Cornell University
Cornell University, Medical Center
Cryobiology Laboratory

Dartmouth College
Delaware, University of
Denver, University of
De Pauw University
Dight Institute for Human Genetics
Dillard University
Drew University
Duke University
Duke University, Medical Center

East Carolina University
Eastern Virginia Medical School
Eastman Kodak Co.
ECO Research Inc.
Ecosystems Laboratory
Eisenhower College
Emory University
Environmental Research Laboratory
ETEC Corp.
Ethicon Research Foundation

Florida, University of
Florida State University
Frederick Cancer Research Center

General Electric Corporate Research & Development
Georgetown University
Georgia, University of
Georgia, Medical College of
Good Samaritan Hospital & Medical Center
W. L. Gorg Associates
Goucher College

Hahnemann Medical College
Hamamatsu Systems, Inc.
Hamilton College
Hampshire College
Harbor Branch Foundation
Harvard Medical School
Harvard School of Public Health
Harvard University
Hawaii, University of, Kewalo Marine Laboratory
Hawaii, University of, School of Medicine
Hofstra University
Holy Cross College
Hopkins Marine Center
Houston, University of
Howard University
Howard University, College of Medicine
Humboldt State University
Hutchinson Cancer Center

Illinois, University of
Illinois, University of, College of Medicine
Illinois Institute of Technology
Indiana University
Indiana University, School of Medicine

Institute for Cancer Research, The
Iowa, University of
Iowa, University of, Hospitals & Clinics
Iowa State University

JEOL-USA, Inc.
John B. Pierce Foundation Laboratory
Johns Hopkins University, The
Johns Hopkins University, The School of Hygiene
Johns Hopkins University, The School of Medicine

Kansas, University of
Kentucky, University of
Kresge Eye Institute

Laboratory of Biophysics, NINCDS-NIH
Laboratory for Comparative Biochemistry
Laboratory Procedures Northwest
Laboratory of Sensory Sciences, Honolulu
Lafayette College
Lederle Laboratory
Lehigh University
E. Leitz, Inc.
LKB Instruments, Inc.
Louisiana State University
Louisville, University of
Loyola College
Luther College

Macalester College
Manhattan College
Marine Biological Laboratory
Marthas Vineyard Shellfish Group
Maryland, University of
Maryland, University of, School of Medicine
Massachusetts, University of, Amherst
Massachusetts, University of, Boston
Massachusetts Audubon
Massachusetts Eye & Ear Infirmery
Massachusetts General Hospital
Massachusetts Institute of Technology
Massachusetts State Lobster Hatchery
Merck, Sharp, & Dohme Research Laboratories
Mercy College
Merrell Research Center
Miami, University of
Miami, University of, School of Medicine
Michigan, University of
Michigan State University
Middlebury College
Millersville State College
Milton S. Hershey Medical Center
Minnesota, University of
Minnesota, University of, Medical School
Minnesota Department of Health
Miriam Hospital, The
Mississippi, University of
Monmouth College
Montana, University of
Montefiore Hospital
Morehouse College
Moravian College
Mount Holyoke College
Mount St. Vincent, College of
Muir College
Murray State University

National Animal Disease Center
NASA, Washington
National Bureau of Standards
National Cancer Institution
National Institute of Environmental Health Sciences
National Institute of Mental Health/NIH
National Institute of Health
National Marine Fisheries Service/NOAA
Naval Dental Research Institute
Naval Regional Medical Center
Naval Submarine Medical Research Laboratory
Nebraska, University of
New England Nuclear Corporation
New Hampshire, University of
New York, City University of, Brooklyn College
New York, City University of, City College
New York, City University of, Herbert Lehman College
New York, City University of, Hunter College
New York, City University of, Medgar Evers College
New York, State University of, Binghamton
New York, State University of, Buffalo
New York, State University of, Downstate Medical Center
New York, State University of, Oneonta
New York, State University of, Stony Brook
New York, State University of, Syracuse
New York, State University of, Upstate Medical Center
New York University
New York University Medical Center
New York University School of Medicine
Nikon, Inc.
North Carolina, University of, Chapel Hill
North Carolina, University of, Raleigh
North Carolina, University of, School of Medicine
North Carolina State University
Northeastern Illinois University
Northport Veterans Administration Medical Center
Northwestern University
Northwestern University, Medical School
Notra Dame, University of

Oak Ridge National Laboratory
Oakland University
Oberlin College
Oklahoma State University

Olympus Corporation of America
Opti-Quip, Inc.
Oregon, University of
Oregon State University
Our Lady of the Lourdes Hospital

Pacific, University of
Pacific Optical Specialists
Pennsylvania, University of
Pennsylvania, University of, School of Medicine
Pennsylvania Medical College
Pennsylvania State University
Pergamon Press
Perkin-Elmer Corporation
Pittsburgh, University of
Plum Island Animal Disease Center
Polaroid Corporation
Population Council, The
Princeton University
Proctor Community Hospital
Public Health Research Institute
Purdue University

Rensselaer Polytechnic Institute
Rhode Island, University of
Rhode Island Hospital
Rice University
Rochester, University of
Rockefeller Foundation
Rockefeller University, The
Roswell Park Memorial Institute
Rush Medical College
Rush University
Rutgers College
Rutgers—The State University of New Jersey
Rutgers University Medical School

St. Andrews University
St. Barabas Medical Center
St. Catherine, College of
St. Elizabeth, College of
St. Joseph Medical Center
St. Louis University
St. Rose, College of
St. Vincent's Hospital
Sangamon State University
Santa Fe, College of
Savannah River Ecology Program
Scheie Eye Institute
Scripps Institute of Oceanography
Seton Hill College
Sherbrooke, University of
Sidney Farber Cancer Institute
Simmons College
Sloan-Kettering Institute
Smith College
Smithsonian Institution
South Florida, University of
Southern Illinois University
Southwest Texas State University
Stanford University
State of New York, Department of Health
Stauffer Chemical Co.
Stephens College
Strang Clinic
Suffolk University
Swarthmore College
Sweetbriar College
Swift Instruments, Inc.
Syracuse University
Syracuse University, Institute for Sensory Research

Temple University Medical School
Tennessee, University of
Tennessee State University
Texas, University of, Arlington
Texas, University of, Austin
Texas, University of, Galveston
Texas, University of, Medical Branch
Texas, University of, Southwestern Medical School
Texas Christian University
Texas Technical University
Toledo Path, Inc.
Tousimis Research Corporation
Trinity College
Tufts University
Tulane Medical Center
Tulane University

Union University, Albany Medical College of
United States Department of Agriculture
United States Geological Survey
Utah, University of

Vanderbilt University
Vanderbilt University, School of Medicine
Venus Scientific, Inc.
Vermont, University of
Veterans Administration Hospital, Brooklyn
Veterans Administration Medical Center of Boston
Veterans Administration Medical & Regional Office Center
Virginia, University of
Virginia Polytechnic Institute

Washington, University of
Washington College
Washington University
Washington University, School of Dentistry
Washington University, School of Medicine
Wayne State University
Wayne State University, School of Medicine
Wellesley College
Wesleyan University
Wheaton College
Williams College
Wisconsin, University of
Wistar Institute
Wistar Institute for Anatomy & Biology
Woods Hole Oceanographic Institution
Wooster College

Worcester Foundation for Experimental Biology
Worcester Polytechnic Institute
WPVI–TV
Yale University
Yale University, School of Medicine
Yale University, School of Forestry & Environmental Studies

FOREIGN

Alberta, University of, Canada
Amsterdam, University of, Netherlands
Arecibo Laboratories, Puerto Rico
Athens, University of, Medical School, Greece
Basel, The University of, Biocenter, Switzerland
Biocenter Laboratory, Ahmedabad, India
British Columbia, University of, Canada
Calgary, University of, Canada
Cambridge, University of, England, U. K.
Caribbean Marine Biological Institute, Netherlands
Centro de Biofisica y Bioquimica, Caracas, Venezuela
Centre for Overseas Pest Research, Kensington, London, England, U. K.
City University, London, England, U. K.
Colegio Universitario, Alicante, Spain
Dalhousie University, Canada
Edinburgh, University of, Scotland, U. K.
Escuela Tecnica Superior de Ingenieros Industriales de Barcelona, Spain
Freien Universität, Institut für Tierphysiologie, Berlin, West Germany
Friedrich Miescher-Institut, Switzerland
Gran Mariscalde Ayacucho, Venezuela
Grenoble, University of, France
Guam, University of
Hebrew University, The, of Jerusalem, Israel
Ibadan, University of, Nigeria
Imperial College of Science & Technology, London, England, U. K.
Institut Pasteur, Paris, France
Instituto de Biologia del Desarrollo
Instituto Venezolano de Investigaciones, Venezuela
Kings College, London, England, U. K.
Laval, University of, Canada
Leeds, University of, England, U. K.
Manitoba, University of, Canada
McGill University, Canada
McMaster University, Canada
Medical Research Council, England, U. K.
Memorial University of Newfoundland, Canada
Nagoya University, Japan
North Wales, University of, Wales, U. K.
Ottawa, University of Canada
Philippines, University of
Philippines, University of, College of Medicine
Physical Research Laboratory, India
Queen Mary College, University of London, England, U. K.
Queens University, Canada
Saint Andrews University, Gatty Marine Laboratory, Scotland, U. K.
Scarborough College, Canada
Sheffield, University of, England, U. K.
Southampton General Hospital, England, U. K.
Southampton, University of, England, U. K.
Stazione Zoologici di Napoli, Italy
Sussex University, England, U. K.
Tel-Aviv University, Israel
Tokyo Medical & Dental University, Japan
Toronto, University of, Canada
Universidad Nacional de Tucuman, Argentina
University College, London, England, U. K.
Venezuelan Institute for Scientific Research
Waterloo, University of, Canada
Western Ontario, University of, Canada

XIII. LABORATORY SUPPORT STAFF

Including Persons Who Joined or Left the Staff During 1979

GROSS, PAUL R., President and Director
SMITH, HOMER P., General Manager

ADMINISTRATION

Controller's Office

CASEY, EDWARD G., Controller
CAMPBELL, RUTH B.
DAVIS, DORIS C.
ELLIS, NANCY L.
HOBBS, ROGER W., JR.
HOWARD, JOAN E.
SEMINO, SUZANNE J.

Director's Office

GROSS, PAUL R., President and Director
LAMBERT, DONNA L.
MORRIS, MAUREEN M.

Education Office

MASER, MORTON D, Assistant Director for Educational and Research Services
LEIGHTON, JANE L.
SAWDO, PAMELA V.

General Manager's Office

SMITH, HOMER P., General Manager
BUTZ, FLORENCE S.
GEGGATT, AGNES L.
JOHNSON, FRANCES N.

Public Relations Office

MAHER, ANNE CAMILLE, Public Relations Officer
WYE, DENICE J.

Biological Bulletin

METZ, CHARLES B., Managing Editor
RUSSELL-HUNTER, W. D., Managing Editor
MORSE, LAURIE; SCHWARTZ, SUSAN

BUILDINGS AND GROUNDS

GUNNING, A. ROBERT, Superintendent
BOURGOIN, LEE E.
BRODERICK, MADELINE H.
DONOHOE, JOSEPH E.
ENOS, GLENN R.
FUGLISTER, CHARLES K.
GEGGATT, RICHARD E., JR.
GIBBONS, ROBERTO G.
GONSALVES, WALTER W., JR.
HOBBS, ROGER W., JR.
KLEINDINST, THOMAS N.
KUIL, ELISABETH
LEHY, DONALD B.
LEWIS, RALPH H.
LOCHHEAD, WILLIAM M.
LOVERING, RICHARD A.
LUNN, ALAN G.
MACLEOD, JOHN B.
MAURER, JOHN E.
MILLS, STEPHEN A.
MOORE, MORGAN
ST. JEAN, SIMONE
SEARS, CLAYTON
SMART, MERILYN A.
THRASHER, FREDERICK
WARD, FREDERICK
WHITTAKER, WILLIAM

GRAY MUSEUM

TIFFNEY, WESLEY N., Curator
BUSH, LOUISE F.
MONTIERO, EVA S.
MOUL, EDWIN T.
WIER, SUSAN J.

LIBRARY

FESSENDEN, JANE, Librarian
ASHMORE, JUDITH A.
BAILY, REBECCA J.
FITZGERALD, DAVID J.
FRANK, CHARLOTTE F.
GRICE, JOAN H.
IRVING, LYNNE A.
JOSEPH, E. LENORA
KARALEKAS, HOLLY E.
SWAIN, LAUREL
WEST, G. ALEXANDER
WHITE, M. ANN

MARINE RESOURCES

VALOIS, JOHN J., Manager
ENOS, EDWARD G., JR.
ENOS, JOYCE
HEBDEN, ROBERT M.
LANE, HOWARD A.
LAWDAY, LEWIS M.
TASSINARI, EUGENE
TRAPASSO, BRUNO
VARAO, JOHN

RESEARCH SERVICES

MASER, MORTON D, Assistant Director for Educational and Research Services
ANDRADE, JULIE A.
ANTHONY, THOMAS R.
BARNES, FRANKLIN D.
BARNES, JOHN S.
CARRINGTON, CATHY A.
EBERHARD, CAROL A.
GOLDER, LINDA M.
GOLDER, ROBERT J.
MARTIN, LOWELL V.
SILVA, MARK
SYLVIA, FRANK E.

SUMMER SUPPORT STAFF EMPLOYEES

ASHLEY, MARY
ASHMORE, JILL
BARTH, MICHAEL
BELLINGHAM, JAY
BRODEUR, JANET
BURNETT, LYNN
CARRUTH, SHARON
CORNELL-BELL, ANN
FEDDE, ELEANOR
GALVIN, REBECCA
HALEY, BETH
HANSEN, ERIC
HANSON, LISA
IRISH, BRADFORD
KUKI, ATSUO
LUNN, JEFFREY
MAINWARING, DANA
MARTIN, DANIEL
MASER, JILL
MASLAND, MARY
MCKINNON, JANE
MILDVAN, HEATHER
MOORE, S. THOMSON
NORDGREN, MARCIA
OFFENBACH, NANCY
PARKER, TIMOTHY
PARPART, LAURA
PETERSON, PHILIP
PHILLIPS, CARL, JR.
RYAN, DANIEL
SULLIVAN, GAIL
TRAVIS, MARK
UPTON, EDWARD
VALOIS, FRANCIS
WALKER, ALLEN
WEISS, NOAH
WEST, HILLARY
WETZEL, ERNEST
WHITTAKER, WILLIAM A.
YOUNG, SARA

Reference: *Biol. Bull.* **159**: 102–116. (August, 1980)

DEVELOPMENT OF THE CILIATURE PATTERN ON THE EMBRYO OF THE SQUID *LOLIGO PEALEI*: A SCANNING ELECTRON MICROSCOPE STUDY

JOHN M. ARNOLD AND LOIS D. WILLIAMS-ARNOLD

Kewalo Marine Laboratory, Pacific Biomedical Research Center, 41 Ahui St.,
Honolulu, Hawaii 96813; and Marine Biological Laboratory,
Woods Hole, Massachusetts, 02543

One feature of embryonic development that seems almost universal is the appearance of ciliated cells on the epithelium of developing embryos. These ciliated cells may be scattered over the embryonic surface or may exist in patterns specific enough to have taxonomic or evolutionary significance. Although there is great interest in the biochemical basis of movement in cilia and flagella, there seems to be little current interest in the significance to the embryo of the motion generated by ciliated cells. This paper describes the development of the ciliature pattern on the embryos of *Loligo pealei* and speculates on the function and significance of the various types of ciliated cells found at different times during development.

Relatively few scanning electron microscope (SEM) studies have been made on the patterns of ciliature of developing embryos and most of these have been done on amphibians. Kessel *et al.* (1974) found in *Rana pipiens* that ciliated cells appeared in the future epidermis during the neural plate stage and that the number of ciliated cells increased greatly during development of the neural folds. By the tailbud stage ciliated cells were widely distributed over the entire embryonic surface but in post-hatching larvae (Stage 24) the cilia began to regress, apparently by resorption. They speculated the ciliated cells functioned in embryonic movement, facilitated respiration, and were important in the movement of mucus across the embryo.

Billett and Courtenay (1973) and Landstrom (1977) have examined development of the ciliature of *Ambystoma mexicanum* embryos. Ciliated cells first appear at the one-somite stage. By the time four to eight somites are formed, one in three surface cells on the flank are ciliated. These ciliated cells are always separated from each other by non-ciliated cells. There is a weak anterior-posterior gradient in number of the ciliated cells but some ciliated cells are present on every surface region. Since the ciliated cells appear first in slight depressions or pits, Billett and Courtenay (1973) speculate that these cells may be moving up from below the epidermal layer. Smith *et al.* (1976) treated *Xenopus* embryos with lithium to produce exogastrulae and found that appearance of ciliated cells was delayed, while Grunz *et al.* (1975) found vegetalizing agents inhibited formation of ciliated cells in isolated *Triturus* ectoderm.

Bergquist *et al.* (1977) found "club-footed" cilia in several species of *Demospongiae*. These cilia have terminal expansions several times the diameter of the ciliary shaft. The expansions are sometimes flattened into paddles and contain irregular membranous vesicles and dense material in addition to a typical axoneme. They did not assign a special function to these "club-footed" cilia but did speculate that such an expansion could cause more efficient movement of the larval sponges.

Sundermann-Meister (1978), using light microscopy, described a new type of ciliated cell from late embryos and juvenile *Loligo vulgaris*. Cilia of these cells are fused into a single common rod. These rods project from surfaces of the fin, mantle, funnel apparatus, head, arms, and suckers. This cilia-rod does not appear to beat and may be a mechanoreceptor.

In this paper we describe four different types of ciliated cells and discuss their function(s) in relationship to the embryonic metabolism.

Materials and Methods

Embryos were obtained from adult *Loligo pealei* using methods described by Arnold (1962); the embryonic stages referred to are those of Arnold (1965). For scanning electron microscopy, embryos were fixed at room temperature in 2% glutaraldehyde in sea water buffered in pH 7.4 with collidine-HCl for 30–40 min. After a 30 min rinse in collidine-HCl buffered sea water, the embryos were post-fixed in 1% OsO_4 in sea water adjusted to pH 7.4 with collidine for 30–45 min. The embryos were then dehydrated through a graded series of acetone, and critical-point dried with CO_2. They were then mounted on metal stubs and coated with gold in a vacuum evaporator with a rotating stage or with a sputterer. Some cracking of the surface occurred because of the massive amount of yolk in the embryo and the hemal sinuses.

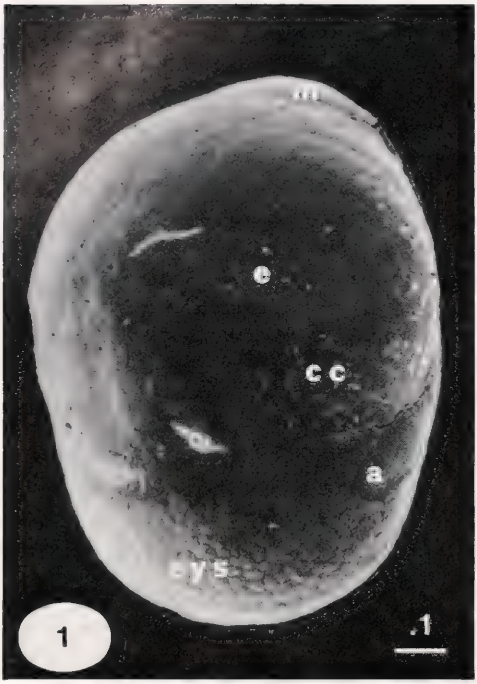
Fixation for transmission electron microscopy was done in 1% OsO_4 with Palade's buffer at pH 6.8–7.0 for 20–30 min at room temperature. The embryos were dehydrated with a graded ethanol series and embedded in Luft (1961) Epon.

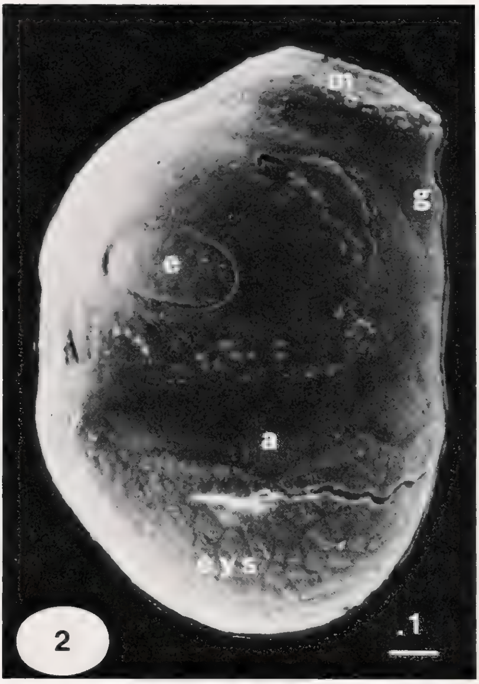
Because many of the low magnification micrographs had backgrounds which distracted from and confused the image of the embryo itself, in several instances (Figs. 1, 2, 7, 8, 11–16, 20–22, 24, 25, and 27) the image of the embryo was carefully cut out of the micrograph and mounted on an artificial background of black photographic paper.

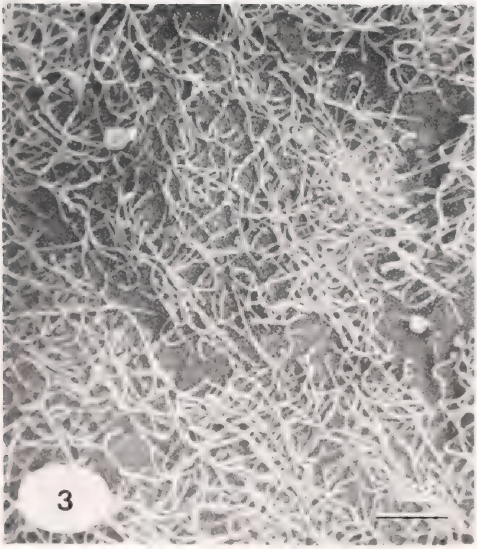
Measuring the speed and direction of particles moved by the ciliated cells in living embryos was found impractical because of heterogeneous distribution of some of the ciliated cells. It was possible, however, to plot the general direction of particle flow. This is expressed with arrows on some micrographs. On the external yolk sac where the ciliated cells are quite uniform, it was possible to measure particle movement as 1.18 mm/sec. But this cannot be compared to the other types of ciliated cells.

Results

Ciliated cells first appear at the same time as or slightly before the organ primordia (Stage 17). They are first evident on the future external yolk sac in a pavement-like arrangement of polygonal flattened cells on the lower $\frac{1}{2}$–$\frac{1}{3}$ of the embryo (Figs. 1 and 2). All cells of the external yolk sac are ciliated with no intervening glandular or other epithelial cells (Fig. 3). The margin of each cell has an area of variable width free of cilia. The cell surface margin is irregular and not covered with microvilli or other surface protrusions. Directly beneath the cells of the external yolk sac, a large hemal space develops. For a time, this is the primary circulatory and respiratory organ (Arnold, 1971; Arnold and Williams-Arnold, 1976). As the external yolk sac pulses, the individual epithelial cells change shape by stretching or contracting. The individual cilia do not appear







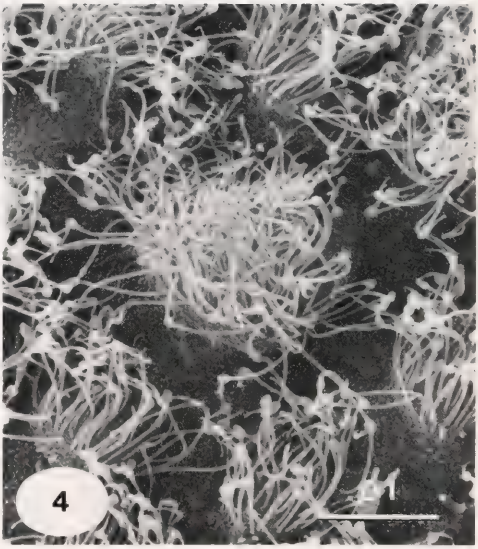
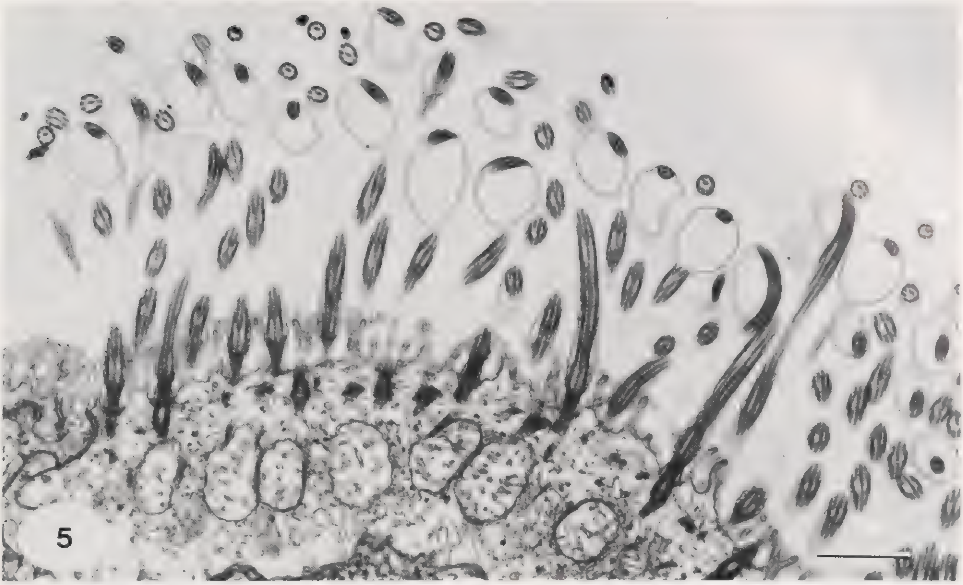
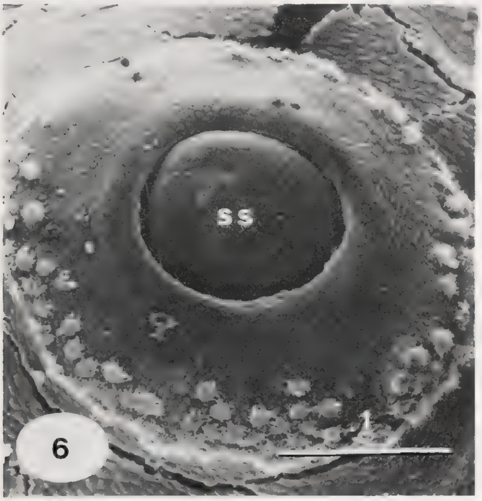


FIGURE 1. Early Stage 18 viewed from the side. The external yolk sac (eys) occupies about ⅓ of this embryo and is covered with flattened ciliated cells. The mantle (m), eye (e), and arm (a) primordia are evident. Ciliated cells (cc) are present below and to the right of the eye. Except where different notations are used, all magnification bars are in mm.

FIGURE 2. Stage 19 viewed from the side. The external yolk sac (eys) abuts directly on the arm region and the individual arm primordia (a) are evident. The eye (e) has begun to form the optic vesicle and one gill primordium (g) is visible. The mantle (m) has paddle-type ciliated cells on the future ventral margin.





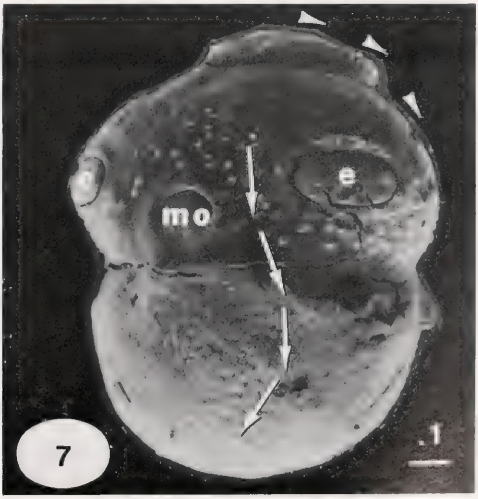


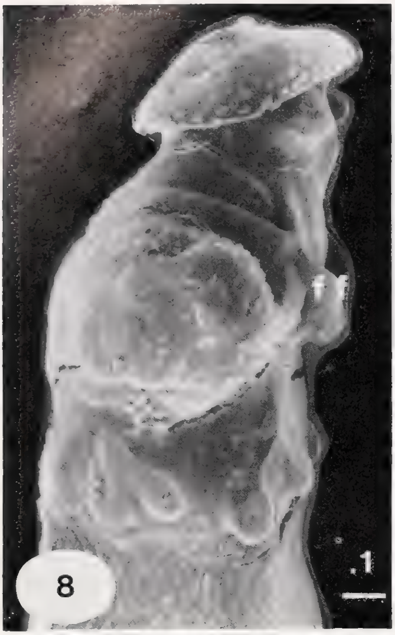
FIGURE 3. Ciliated cells of the external yolk sac at Stage 20. The cells are polygonal in outline and are covered with cilia which appear to beat in an uncoordinated but unidirectional fashion.

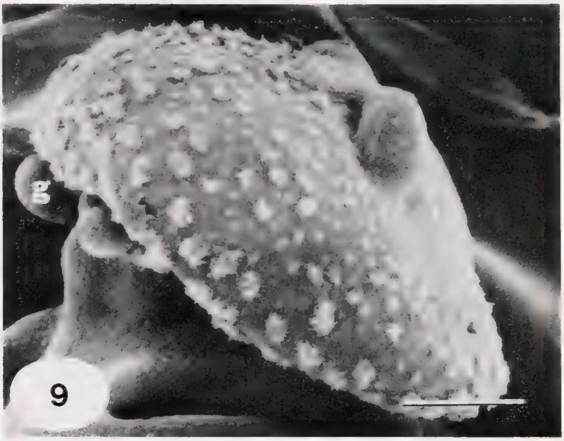
FIGURE 4. Paddle-type ciliated cells on the mantle of the Stage 25 embryo. These cells are almost invariably separated from each other by other cells of the epithelium. The individual cilia are expanded into flattened "paddles" or, more infrequently, spheroidal knobs apically or subapically. Although the beat is unidirectional, it is not synchronous.

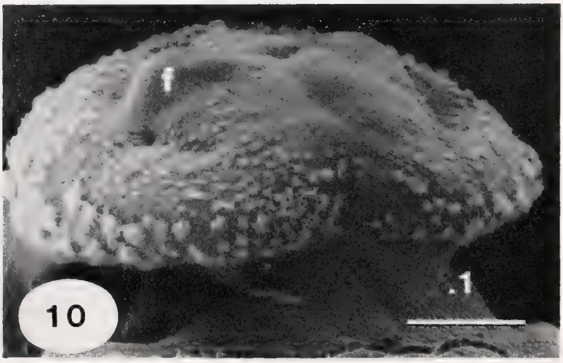
FIGURE 5. Section of one paddle-type ciliated cell. The paddles are composed of an expanded portion of the cilia membrane, which forms an electron transparent vesicle. The axoneme bends sharply within the paddle.

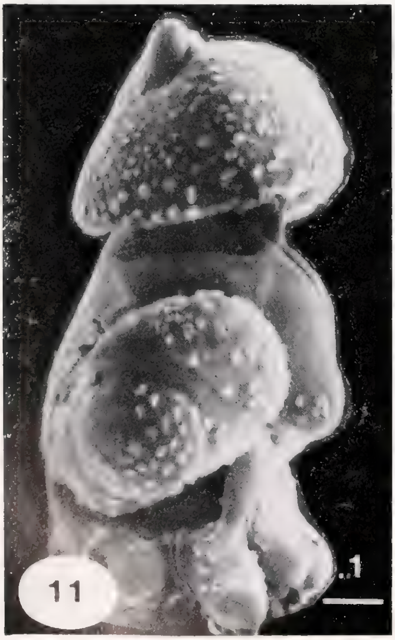
FIGURE 6. Mantle of a Stage 20 embryo during closure of the shell sac (ss). Paddle-type ciliated cells have developed on the future ventral surface (at magnification bar) and sides of the edge of the mantle.

FIGURE 7. Future dorsal surface of the Stage 20 embryo. The number of paddle-type ciliated cells has increased around the eye primordium (e) and above the mouth (mo). The arrows indicate the direction of flow of the chorionic fluid.









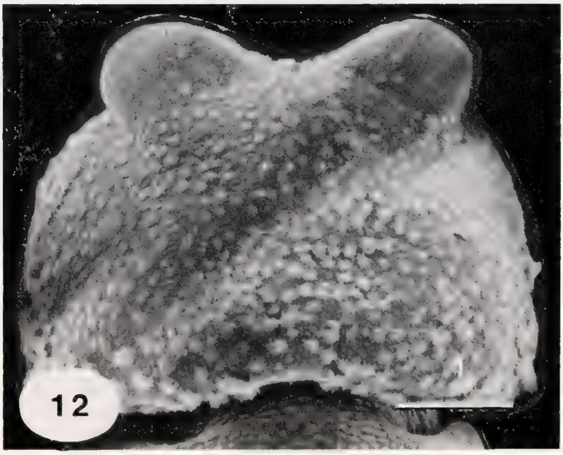
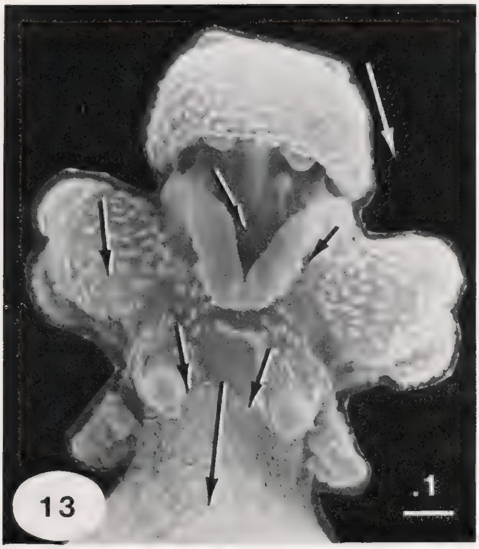
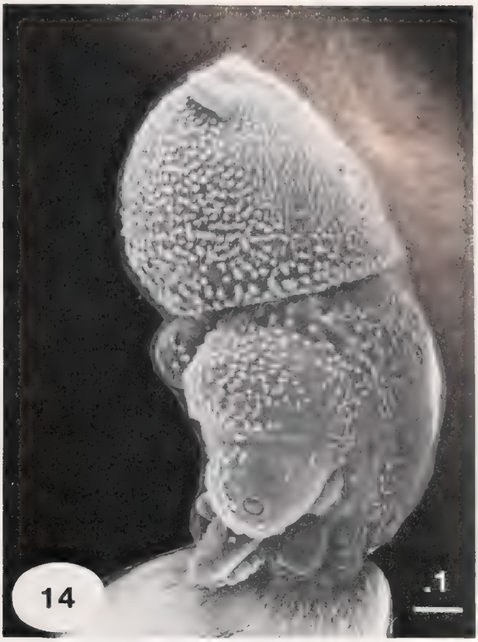
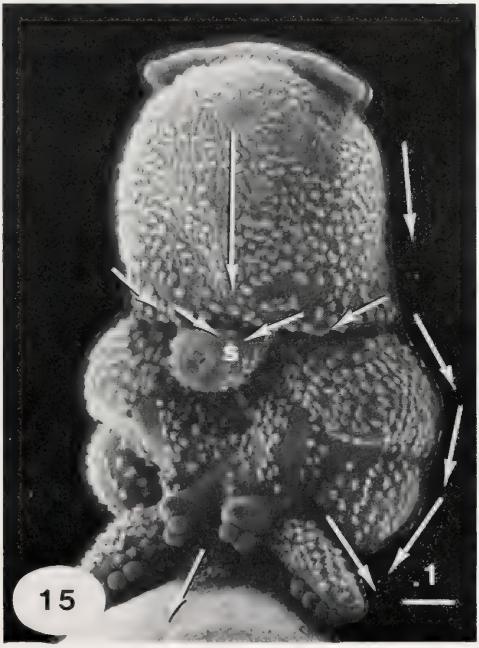


FIGURE 8. Lateral view of a Stage 22 embryo. The external yolk sac has elongated along with the embryo, and cells in the yolk stalk region have changed from polygonal to elongate. A few paddle-type cilia occur on the arms and distal portion of the funnel folds (ff). The number of ciliated cells on the optic stalk has increased.

FIGURE 9. Paddle-type ciliated cells have increased in number and are more or less evenly distributed on the future ventral mantle. The gill primordia (g) and proximal portions of the funnel complex remain unciliated.


13


14


15

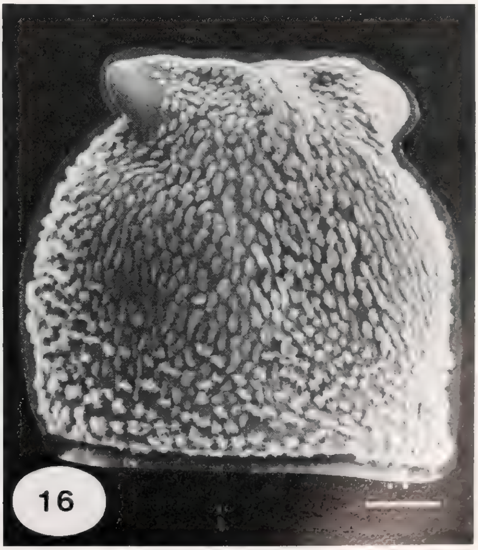

16

FIGURE 10. The dorsal portion of the mantle is becoming ciliated by Stage 22, although the future dorsal midline is last to develop ciliated cells. The fin primordia (f) do not have any cilia on them.

FIGURE 11–13. The number of paddle-type cilia on the mantle has continued to increase and the pattern seems random. Parts of the eye stalk and and some future ventral areas remain unciliated. The arrows indicate the direction of fluid flow. See text for details.

FIGURES 14–16. At Stage 26, there are regions of the head which lack ciliated cells, notably above the eye and on the future dorsal head. A major change has occurred on the mantle with the appearance of "uniform-type" ciliated cells. These cells tend to form lines in which all the cilia have unidirectional and synchronous beat. (s = siphon). See text for details. On Figure 15 the arrows indicate the direction of movement of the chorionic fluid.

to have any unusual features. In SEM there is no evidence of synchrony of beat, but in living embryos, the cilia beat toward the vegetal pole of the embryo.

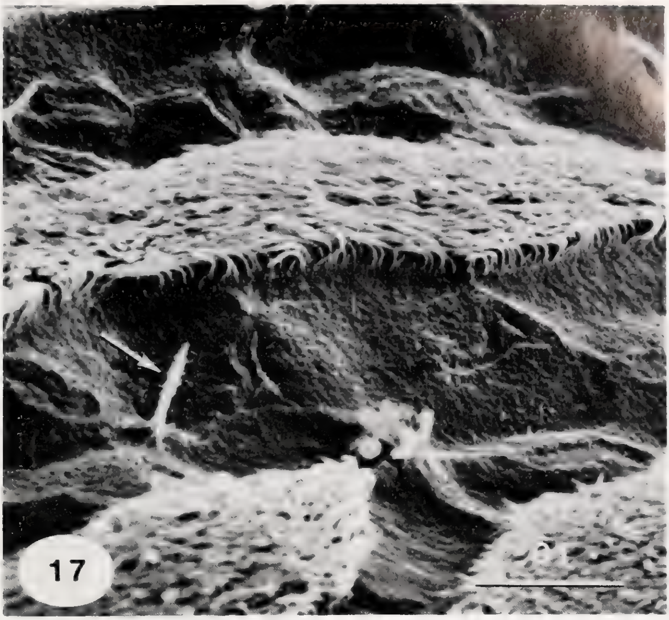
A second type of ciliated cell appears at about Stages 17 and 18, first on the ventral and anterior edge of the eye primordia, then on the future ventral margin of the mantle (Figs. 1 and 2). These cells are large, flattened, and separated from each other by non-ciliated neighboring cells (Fig. 4). These cilia have a unidirectional but not metachronic beat. They are larger than those on the external yolk sac and have an apical or subapical expansion three to five times the diameter of the rest of the cilium (Fig. 4). In the scanning microscope, this expanded portion frequently appears flattened into a paddle shape, although spheroidal expansions are also occasionally seen. Because of this morphology, they will be referred to as "paddle-type cilia." If the expansion is subapical, the tip of the cilium frequently emerges at an acute angle to the rest of the shaft. Transmission electron micrographs show the expanded portion to be a bleb of "empty" membrane at a bend in the axoneme. The axoneme has the typical "9 + 2" morphology (Fig. 5). In living cells, the paddle appears passive and follows the beat of the cilium. When seen on the return stroke with phase-contrast microscopy, the plane of the paddles is close to and parallel with the cell surface. On the power stroke, it is perpendicular to the angle of thrust.

At Stage 20, the paddle-type ciliated cells are on the sides of the mantle primordium, and the number of ciliated cells and area covered on the eye primordium has increased. They also appear above the mouth primordium (Figs. 6 and 7).

At Stage 22 (Figs. 8–10), the paddle-type ciliated cells occur on the top of the optic stalk and on the future anterior surface of the optic vesicle, while the future posterior of the optic vesicle is unciliated. The mantle has expanded in area and turned downward to begin to cover the developing mantle cavity and the number of ciliated cells has increased accordingly. Most ciliated cells still occur singly, but occasionally two cells are in contact (Fig. 9). The fin primordia remain devoid of ciliated cells. The future dorsal region of the mantle still is temporarily devoid of ciliated cells (Fig. 10). A few paddle-type ciliated cells have appeared on the proximal portion of the arms and on the outer distal end of the funnel primordium (Fig. 8; *cf.* Fig. 11).

The embryo elongates as it grows so that the external yolk sac is attached to the embryonic region proper by an ever-narrowing yolk stalk (Fig. 8). This also causes a change in shape of the individual cells, but the direction of beat apparently is unaffected.

The distribution of cilia on the mantle becomes more uniform as the mantle grows over the future mantle cavity (Stage 23, Figs. 11–13). The edge of the mantle has a fairly continuous line of ciliated cells, and in some instances these cells appear larger with longer individual cilia. The fins do not have ciliated cells except at the margin between them and the base of the future dorsal surface (Fig. 12). The number of paddle-type ciliated cells on the body surface has increased but the organs or parts of the organs which will become incorporated into the mantle cavity remain unciliated (*e.g.*, gills, anal papilla, part of the funnel folds; Fig. 13). The optic stalk has a somewhat uniform distribution of paddle-type ciliated cells except on the future posterior surface of the eye and in a band which lies between the optic stalk proper (Fig. 11). Later in embryonic development, this region gives rise to tissue which covers the optic vesicle and forms the definitive cornea. The tips of the arms and the developing suckers also lack ciliated cells.



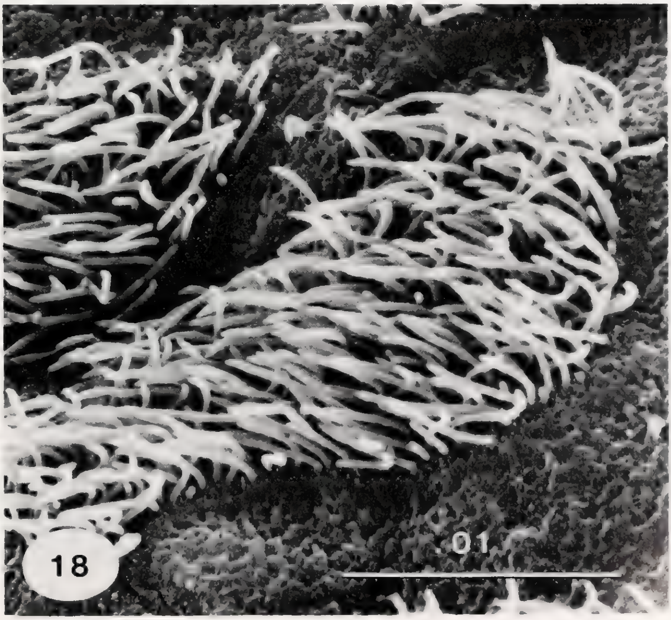
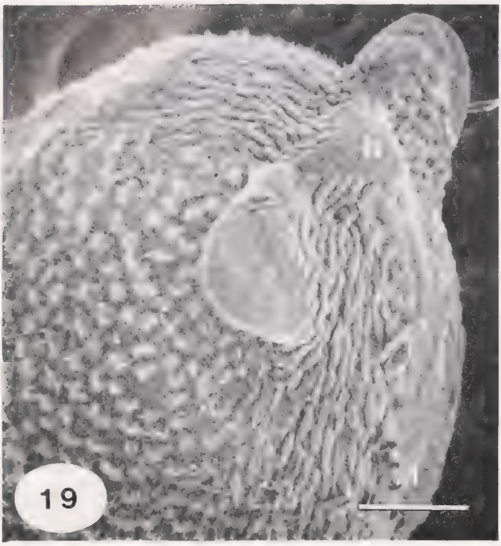
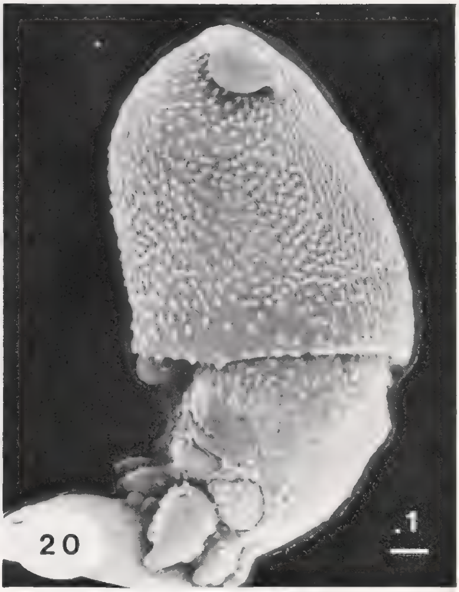


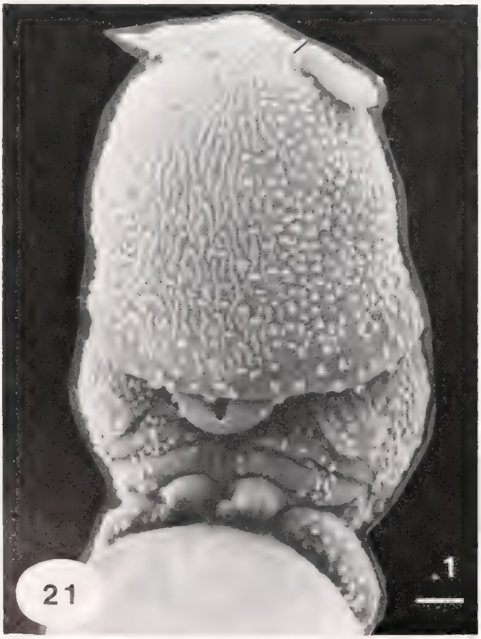
FIGURE 17. The uniform-type ciliated cells beat in a metachronic wave that is synchronized along several aligned cells. The projection in the lower left (arrow) may be a mechanoreceptor.

FIGURE 18. In this developing uniform-type ciliated cell, cilia of several lengths can be seen and the beat, although unidirectional, is only partially synchronous. These cilia are of uniform diameter and end in a blunt taper.

By Stage 26 (Figs. 14–16), the pattern of cilia on the head of the Stage 23 embryo has changed slightly. This includes an increase in the ciliated cells and two lines of paddle-type ciliated cells running from the base of the median pair







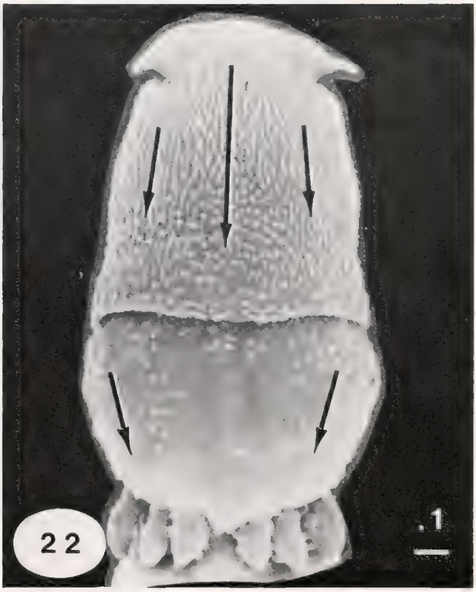


FIGURE 19. The lines of uniform ciliated cells radiate from the developing Hoyle organ (h) at Stage 28. The chorionic fluid is circulated from the future posterior toward the edge of the mantle.

FIGURES 20–22. At Stage 27 the edge of the mantle has a band with relatively few ciliated cells and the lines of uniform-type cilia are more prominent on the future dorsal and future ventral surfaces. On Figure 22, the arrows indicate the general direction of movement of the chorionic fluid.

of arms back toward the optic stalk on the anterior dorsal head (Fig. 14). Ciliated cells are approximately evenly distributed on the posterior dorsal head. The ventral surface of the head is uniformly covered with ciliated cells except between the future ventral-most arms, in a small region over the otocysts and at the junction of the optic lobe region of the optic stalk and the optic vesicle (Fig. 15).

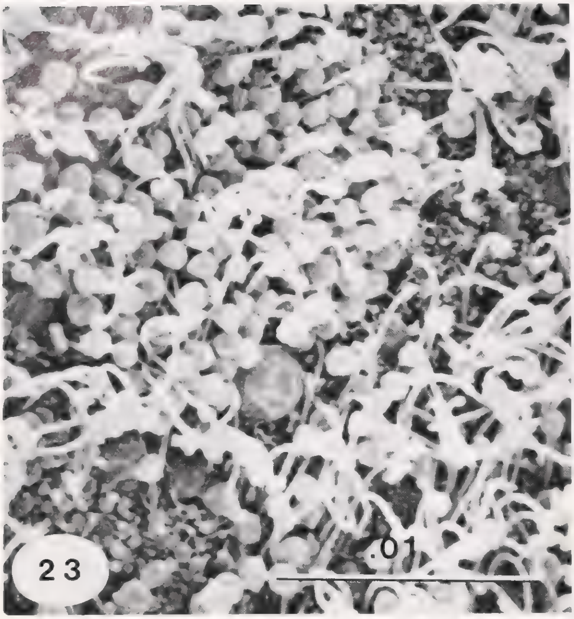
The mantle pattern of ciliature has undergone extensive changes by Stage 28 and a different type of ciliated cell has appeared amid the uniformly distributed paddle-type ciliated cells (Figs. 14–16). These cells are elongate on the anterior-posterior embryonic axis and show a strong tendency to form anterior posterior contact with each other. The cilia are uniform in diameter and taper to a point without an expanded region (Figs. 17 and 18), and will be referred to here as "uniform-type ciliated cells." These cilia beat in a metachronic wave continuing across the cell borders. The cilia on these cells occur close to the margin of the cells. As many as seven cells occur in such an alignment, with groups of four and five common. These lines rarely branch or fork and if they do, it is with one cell. The lines of cells radiate from the future site of the Hoyle organ (hatching gland) which is situated between the fins (Fig. 19). The cilia of the cells do not grow out of the cell simultaneously: In young cells several lengths of cilia can be found and the beat pattern seems irregular (Fig. 18).

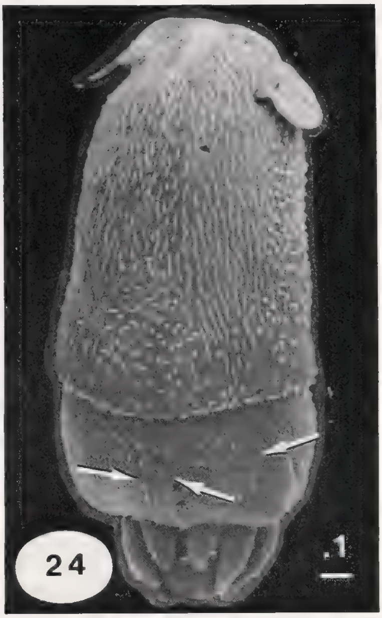
At Stage 27, the mantle elongation has increased so that a band relatively sparse in ciliated cells occurs behind its anterior margin (Figs. 20–22). The ciliature pattern is unchanged except that the ciliated cell-free area of the dorsal head has increased. Aligned groups of uniform-type ciliated cells of the mantle occur in one ventral medial patch and in a "V" on the dorsal surface (Figs. 21 and 22). Between the arms of the "V" and on the sides of the mantle are extensive areas of paddle-type ciliated cells.

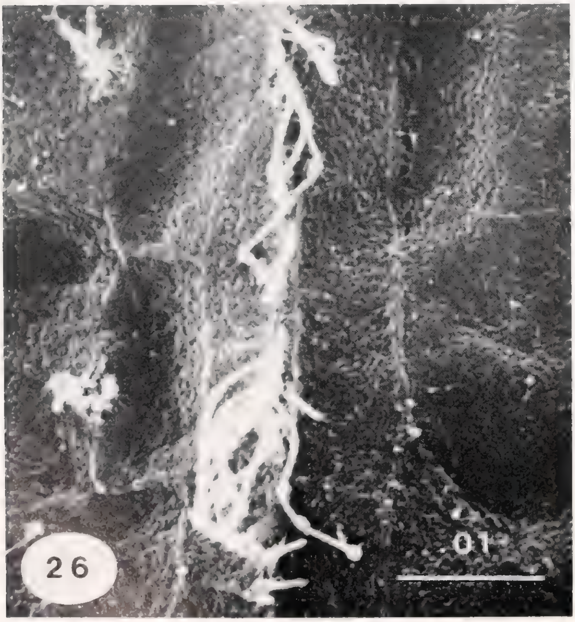
As the embryo grows, the external yolk sac decreases by digestion of yolk and by transfer of its contents into the developing internal yolk sacs. The circumoral musculature constricts the hemal space in the arm region and pulsation of the yolk sac decreases. The cilia of the yolk sac also shorten and become irregular in diameter. They still beat, however, because particles in the chorionic fluid or sea water sweep along the surface of the external yolk sac. High magnification reveals that the apical and subapical portion of these cilia have expanded to flattened discs similar to those of the "paddle-type cilia." Furthermore, these discs frequently have double ridges at their edges, each of axoneme diameter. (Fig. 23).

Before hatching (Stage 29), the external yolk sac continues to decrease in size and the embryo to elongate, causing the yolk-sac cells to become rounded and have a smaller external area. The ciliature pattern on the mantle remains similar to that of the Stage 27 embryo (*cf.* Figs. 21 and 22 with Figs. 24 and 25), but on the dorsal surface of the head, a fourth type of ciliated cell appears (Figs. 24–26). This type occurs as two to four lines of "single-file" ciliated cells running along a central head crest (Fig. 26). Two such rows in the central region of the head are common, but four or occasionally three lines occur. Side-to-side synchronous beat appears along the length of the "single-file" ciliated cell. Exceptionally, the tips of these cilia are apically or subapically expanded; mostly they end in a short tapered tip.

After hatching (Stage 30), an abrupt change occurs in the epithelium of the mantle. Within 24–36 hr, the cells of the mantle begin to slough off and are released into the surrounding sea water as individuals or in small groups (Figs. 27 and 28). The surrounding glandular elements may also be shed. Large gaps appear between the cells bridged by many fine branching extensions of surface material (Figs. 28 and 29).







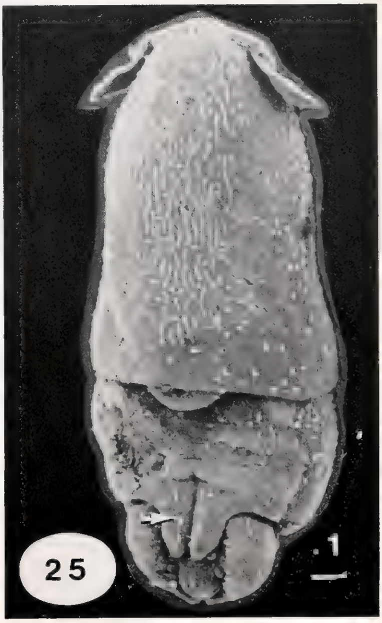
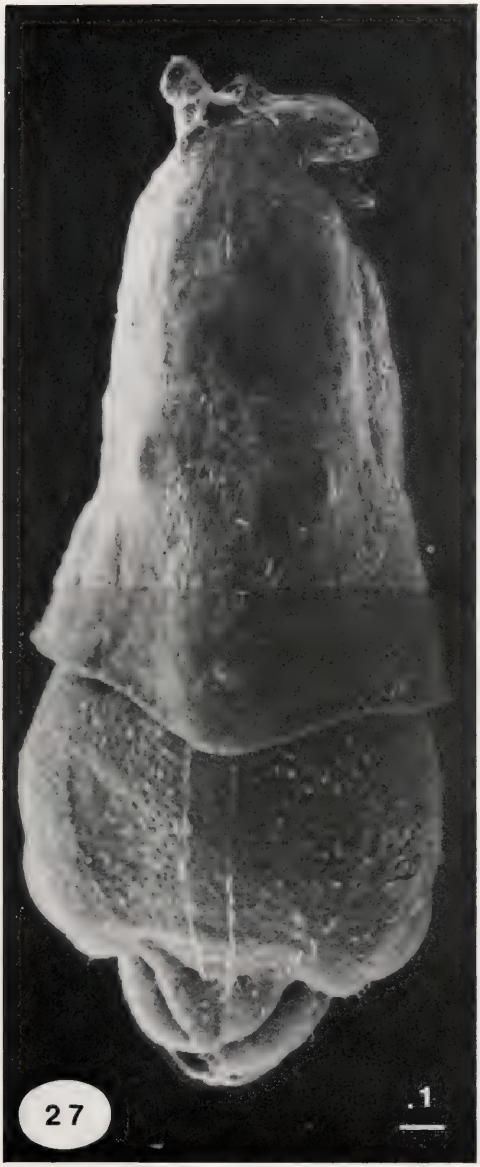
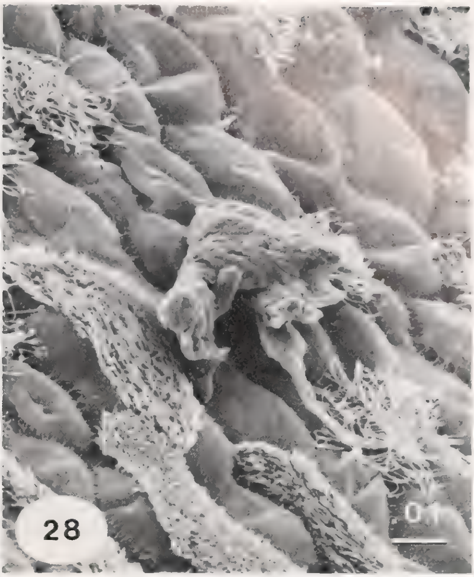


FIGURE 23. After heart and gills assume the respiratory and circulatory functions of the external yolk sac, the sac stops pulsating and the cilia shorten and develop disc-like paddles, possibly by coiling of the axoneme. They continue to beat.

FIGURES 24 and 25. At Stage 29, the ciliature pattern on the mantle has changed little from Stage 27, but "single-file" ciliated cells have appeared on the head (arrows).

FIGURE 26. Higher magnification of the single-file ciliated cells show the cilia arise from a central crest of cells and beat in a lateral wave.





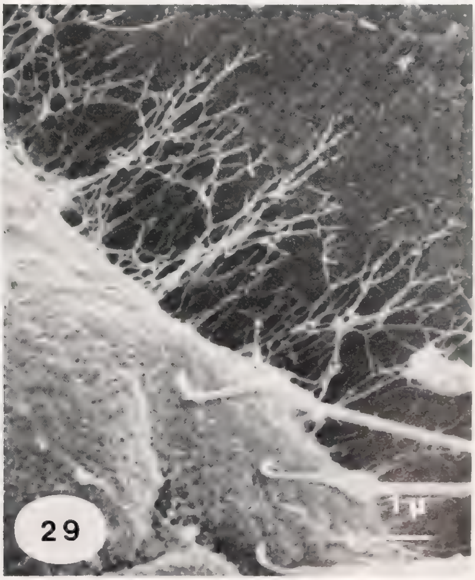


FIGURE 27. After hatching, the ciliated cells of the mantle are shed within 24–36 hr. The ciliated cells of the head are lost later.

FIGURE 28. During shedding, cells separate from each other in longitudinal rows and are lost singly or in small groups. Mucus cells are also probably lost.

FIGURE 29. As the cells of the mantle epithelium pull apart, a meshwork of branching strands which intertwine with cilia becomes apparent. Frequently, cilia can be seen sticking to the surface of non-ciliated cells.

The head ciliature does not similarly change during the 2–3 days we have been able to keep newly hatched juveniles in good health (Fig. 27). Presumably it does so later. The external yolk sac usually is dropped and degenerates soon after hatching. Its cilia are active, however, for a short time after it is cast aside.

Occasionally, non-moving bundles of cilia are observed in the mantle (Fig. 17), or more commonly on the aboral surface of the arms. Presumably these are the mechanoreceptors described by Sundermann-Meister (1978).

DISCUSSION

The question raised by these observations is: Why do three different types of ciliated cells exist to perform similar functions? The time of appearance and placement on the embryo may provide some insight about the function of each type; still, all the questions cannot be answered. The function of the fourth type, the "single-file" cilia, is unknown to us.

Cilia of the external yolk sac arise first. Since the external yolk sac is an embryonic digestive, circulatory, and respiratory organ, its ciliated cells are most probably associated with respiration. The hemal space of the external yolk sac is separated from the chorionic fluid by a thin layer of flexible pavement-like cells. Cilia in this position prevent localized accumulations of carbon dioxide (or excretory products) and facilitate respiratory exchange. Cilia of these cells have a directional beat, but individual cilia do not beat synchronously and unlike amphibian embryos (Kessel *et al.,* 1974; Billett and Courtenay, 1973) the ciliated cells are in contact with each other at their margins. Collectively, these ciliated cells must be quite effective in circulating the chorionic fluid, which in turn is able to exchange metabolic substances through the chorion. Their partial structural degeneration when the gills and branchial hearts take over the respiration and circulation of the developing embryo suggests further that they function primarily in respiratory exchange. Apparently, the development of a disk-like paddle on or near the tips of the cilia later in development is related to the coiling of the axonemes in each individual cilium.

The function of the paddle-type cilia is more obscure. The development of a flattened expanded tip or subapical portion could increase efficiency of the effective stroke. By being flattened, the expanded tip would create little drag in the recovery stroke if the plane of flattening is perpendicular to the plane of bending. The sharp bending of the axoneme where it passes through the expanded portion could be structurally related to strengthening the "paddle" during the effective stroke if the curvature of the band is oriented into the plane of the effective stroke. Observations with phase contrast microscopy confirm this is likely.

Paddle-type ciliated cells always appear isolated from other ciliated cells and first appear on the organ primordia. They are particularly prominent and active. Their placement on the eyes, head, arms, and early mantle suggest that they generally circulate the chorionic fluid past the embryo but also prevent the embryo from directly contacting and possibly attaching to the inside of the chorion. Isolated, non-ciliated embryonic cells do stick to the chorion. Their position on the embryo would also facilitate "cutaneous" respiration. They do not beat synchronously, suggesting autonomous control. The uniform-type ciliated cells have the appearance usually associated with ciliated cells on other tissues and embryos. Their most striking feature is that they have a metachronic beat and synchrony extends from one cell to another in a continuous wave. The alignment and synchrony of the beat of these ciliated cells seems quite effective in moving large volumes of fluid. Since these ciliated cells appear about the same time (Stage 26) as mantle contraction begins and the gills and branchial hearts begin to function, they could increase chorionic fluid circulation. In fact, older embryos continuously tumble inside the ever-expanding chorion. Dechorionated embryos observed in a dish of sea water continuously move posteriorly along the bottom of the dish. Therefore, these rows of aligned synchronous ciliated cells possibly function in enhancing the circulation of the chorionic fluid and movement of the older embryo. This is advantageous to the embryo for respiration and excretion.

The detailed cause(s) of the post-hatching sloughing of the skin of the newly hatched juvenile are unknown. But the phenomenon obviously is related to the breaking of cell-to-cell contacts and cells are lost either as individuals or in small groups. The filamentous material that temporarily connects the cells as they slough is probably the remnant of intercellular substances, since the cell membrane does not appear to be involved. We assume that this sloughing is followed by the development of the slimy skin typical of cephalopods. Because of the difficulties in rearing juvenile squid in the laboratory, we were unable to collect data on this.

The single-file cilia do move, so it would seem unlikely that they function as mechanoreceptors; and since they are not in an enclosed pit, it is unlikely that they are chemosensory. They could possibly function to keep the head free of detritus, but there is never much particulate material in the chorionic fluid and since they are in a single row, it seems unlikely that they could generate much force for this purpose. However, they may move mucus produced by the gland cells of the skin on the head. How long these cilia persist and their ultimate fate is unknown.

The developmental significance of morphologically different ciliated cell types is puzzling. Because they are functionally different and appear at different times during development and on different organs, there must be adequate selection pressure to preserve their separate phenotypes. This is striking since on other embryos, only one recognized motile ciliated cell type must provide all the surface ciliary functions for complete embryonic development. Perhaps these differences between vertebrate embryos and cephalopod embryos reflect their separate evolutionary histories.

The authors wish to thank Drs. Lewis Tilney and Michael Hadfield for reading this manuscript and Mrs. Frances Okimoto and Mrs. Charlotte Daspit for preparing the typescript. This work was supported by grant #PCM 7619301 from the National Science Foundation and the *Teuthis obscura* fund. Dr. Virginia Peters taught us many of the techniques used here.

SUMMARY

1. Four types of beating ciliated cells, which appear at different times during embryonic development of the squid *Loligo pealei,* are described.

2. The ciliated cells of the external yolk sac first appear during or at onset of organogenesis, as polygonal flattened cells in a pavement-like arrangement. The cilia of these cells are uniform in diameter and end in a blunt or slightly tapered tip. Their beat is asynchronous and uncoordinated, but unidirectional toward the vegetal pole. A large hemal space develops between these cells and the yolk syncytium of the external yolk sac. In later stages of development, when primary respiratory function is assumed by the gills (and probably the skin), the

cilia on those cells develop a flattened paddle at or near their tips, which apparently is associated with coiling of the axonemes within them.

3. Paddle-type ciliated cells develop on the embryonic body proper and are always isolated from each other except at the anterior edges of the mantle. These cilia beat unidirectionally and asynchronously. The axis of the paddle is parallel to the cell surface on the return stroke, thus offering little resistance to the bending of the cilia, and is perpendicular to the direction of the power stroke, thereby increasing the effectiveness of the power stroke. Cells of this type appear on the mantle and head in patches, but not in areas to be covered by other tissues such as the mantle.

4. At or just before Stage 26, a third type of ciliated cell appears in rows on the mantle. The individual cilia are uniform in diameter and end in a tapered tip. The cilia on these cells beat in a metachronic wave synchronized among several cells aligned in rows. These lines of "uniform-type" ciliated cells are apparently very effective because the embryos begin to swim and tumble actively in the chorionic fluid and circulate it rapidly. This probably enhances respiration and prevents the embryo from sticking to the inner surface of the chorion.

5. A fourth type of beating ciliated cell, the "single-file" ciliated cell, appears on the head and ventral arms of the late Stage 28 or early Stage 29 embryo. The cilia of these cells are aligned in single file on the anterior-posterior axis and beat in a synchronized side-to-side wave. The function of these cells is unknown.

6. At hatching, the entire mantle epithelium degenerates and is sloughed off. Presumably, the epithelium of the head is also shed, but was unobserved by us.

LITERATURE CITED

Arnold, J. M., 1962. Mating behavior and social structure in *Loligo pealei*. *Biol. Bull.*, **123**: 53–57.

Arnold, J. M., 1965. Normal embryonic stages of the squid *Loligo pealei* (Lesueur). *Biol. Bull.*, **128**: 24–32.

Arnold, J. M., 1971. Cephalopods. Pp. 265–311 in G. Reverberi, Ed., in *Experimental embryology of marine and fresh water invertebrates.* North-Holland Publishing Co.

Arnold, J. M., and L. D. Williams-Arnold, 1976. The egg cortex problem as seen through the squid eye. *Am. Zool.*, **16**: 421–446.

Bergquist, P. R., C. R. Green, M. E. Sinclair, and H. S. Roberts, 1977. The morphology of cilia in sponge larvae. *Tissue & Cell*, **9**: 179–184.

Billett, F. S., and T. H. Courtenay, 1973. A stereoscan study of the origin of ciliated cells in the embryonic epidermis of *Ambystoma mexicanum*. *J. Embryol. Exp. Morphol.*, **29**: 549–558.

Grunz, H., A.-M. Multier-Lajous, R. Herbst, and G. Arkenberg, 1975. The differentiation of isolated amphibian ectoderm with or without treatment with an inductor. *Wilhelm Roux Arch. Entwickl.-Mech. Org.*, **178**: 277–284.

Kessel, R. G., H. W. Beams, and C. Y. Shih, 1974. The origin, distribution, and disappearance of surface cilia during embryonic development of *Rana pipiens* as revealed by scanning electron microscopy. *Am. J. Anat.*, **141**: 341–360.

Landstrom, U., 1977. On the differentiation of prospective ectoderm to a ciliated cell pattern in embryos of *Ambystoma mexicanum*. *J. Embryol. Exp. Morphol.*, **41**: 23–32.

Luft, J. H., 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.*, **9**: 409–414.

Smith, J. L., J. C. Osborn, and M. Stanisstreet, 1976. Scanning electron microscopy of lithium-induced exogastrulae of *Xenopus laevis*. *J. Embryol. Exp. Morphol.*, **36**: 513–522.

Sundermann-Meister, G., 1978. Ein neuertyp von cilienzellen in der Haut von spätembryonalen und juvenilen *Loligo vulgaris*. (Mollusca, Cephalopoda). *Zool. Jb. Anat.*, **99**: 493–499.

Reference: *Biol. Bull.* **159**: 117–134. (August, 1980)

INTERSPECIFIC AND INTRASPECIFIC ACRORHAGIAL AGGRESSIVE BEHAVIOR AMONG SEA ANEMONES: A RECOGNITION OF SELF AND NOT–SELF

CHARLES H. BIGGER[1]

Department of Biological Science, Florida State University, Tallahassee, Florida 32306

Coelenterates have long been considered primitive or simple animals. Although some aspects have been known for a long time, coelenterate behavior and its physiological and morphological bases are still poorly understood. Studies have now shown coelenterates to be capable of a variety of fairly complex behaviors, including aggression (Pantin, 1952; Francis, 1973b; Ross, 1974).

All sea anemones possess tentacles, the interior of which are continuous with the digestive cavity (coelenteron). In addition, some species have other "hollow" structures, such as the acrorhagi. Although acrorhagi were first described by Rapp in 1829, as small, light-blue buttons on the rim of the disc of *Actinia mesembryanthemum* (= *Actinia equina*), the function of these structures remained a mystery until the middle of this century.

Based on morphological evidence alone, functions attributed to the acrorhagi included photoreception (Hollard, 1851; Duncan, 1874), sensitivity to touch (Korotneff, 1876), and defense (Gosse, 1860). Neither the Dixons (1891), who gave one of the first accounts of the behavior associated with acrorhagi, nor Walton (1910) perceived the importance of their observations concerning the acrorhagi. It remained for Abel (1954) to recognize the significance of and more completely describe the acrorhagial response of sea anemones.

Abel (1954), Bonnin (1964), Francis (1973b), Bigger (1976), Williams (1978), and Brace and Pavey (1978) have reported the same basic acrorhagial behavior in five species of sea anemones. After an acrorhagi-bearing animal touches some conspecifics, or certain other coelenterates, the acrorhagi in the area of contact swell and elongate. The expanded acrorhagi are placed on the other animal, withdrawn, and then the application process is repeated. Pieces of the acrorhagial ectoderm (acrorhagial peels) remain on the target animal. Within as little as 20 min (Bonnin, 1964), the tissue of the target animal receiving the peel exhibits signs of necrosis. The acrorhagial response with its directed nature and infliction of injury has been considered an aggressive behavior (Francis, 1973b) and, thus far, has established sea anemones as the "simplest" animals to possess aggressive behavior.

Francis (1973a, 1976) established competition for space as a possible ecological role for the acrorhagial response and demonstrated an intraclonal division of labor between sexual reproduction and acrorhagial aggression in *Anthopleura elegantissima*. Recently the possible relationship of the acrorhagial response to immunocompetence has been discussed (*e.g.*, Hildemann *et al.*, 1975).

This present study further examines the acrorhagial response of four species of sea anemones, two not previously examined, and in particular inquires into the nature of inter- and intraspecific relationships and the question of a possible "immunocompetence."

---

[1] Present Address: Immunogenetics Group, UCLA School of Medicine and Dental Research Institute, Los Angeles, California 90024.

MATERIALS AND METHODS

Animal collection and maintenance

The specimens of *Anthopleura krebsi* used in this study were collected intertidally from a series of rock groins 84 m apart on the sandy substrate of Coquina Beach, Anna Maria Island, Florida. *Anthopleura kerbsi* was identified in accordance with Carlgren and Hedgpeth (1952). The anemones, lining crevices and depressions in the rocks, were collected from aggregations of contacting individuals. Although there were various color patterns, the specimens of *A. krebsi* could be generally classified into two color morphs, red or green. All the acrorhagi tips of an individual were the same color, a shade of red or green. In the laboratory, *A. krebsi* commonly reproduces asexually; thus, the anemones in the field aggregations could have been clonemates. However, the term "groupmates" will be used for the anemones from the same aggregation and the term "clonemates" will be reserved for those anemones known, by direct observation, to have the same genotype as a result of asexual reproduction. The specimens of *A. krebsi* ranged in size from 0.5- to 2-cm pedal-disc diameter.

The anemones were transported to Florida State University where they were maintained, individually, by group, or as clones; in culture dishes, fingerbowls, or filtered aquaria. The natural sea water in the dishes was changed every other day.

Anthopleura xanthogrammica was observed at Victoria, British Columbia, and near the Bamfield Marine Station on Vancouver Island, Canada, during an extremely low tide. *Anthopleura xanthogrammica* tended to be solitary and was found in high-tide pools.

The anemone *Bunodosoma cavernata* was collected from the jetties at the Mayport Naval Station, Mayport Beach, Florida. The anemones occurred alone, or in aggregations among the large rocks of the jetty. When collected, specimens of *B. cavernata* ranged in size from 1.5- to 4.5-cm pedal-disc diameter. They were maintained in 5- or 15-gal filtered aquaria containing natural sea water.

The anemone *Anemonia sargassensis* was collected from the rock jetties of the inlet to St. Andrews Bay, Panama City, Florida. The pedal disc diameter ranged from 1 to 2.5 cm. Although they reproduced asexually by longitudinal fission, clones were not isolated and all specimens of *A. sargassensis* were maintained in the same 15-gal filtered aquarium.

Polyps of the scyphozoan *Cassiopea* from the Florida Keys have been maintained in the laboratory at Florida State University since 1974. After the work reported by Bigger in 1976, medusae were raised from the scyphistomae (polyps) and the species identification was confirmed as *Cassiopea xamachana* (Bigelow, 1900). Clones of *C. xamachana*, including both scyphistomae and medusae, were maintained in separate aquaria making it possible to test the reaction of *A. krebsi* to medusae and scyphistomae from the same clone.

All aquaria were provided with sub-gravel filters and some had additional external glass-wool filters. Illumination was provided by Gro-lux fluorescent lights on a 14:10 light:dark regime. The anemones' diet consisted of pieces of shrimp, beef liver, and newly hatched *Artemia* nauplii from San Francisco Bay Brand eggs; while the *Cassiopea* diet consisted of *Artemia* nauplii. Feeding was stopped 3 days prior to and during an experiment.

Behavior and physiology

Except for filmed interactions, all anemone behavior was viewed through a Wild M5D stereomicroscope. The same rheostat setting on the top-mounted lights was used for all trials to maintain constant lighting conditions. Temperatures during observational periods ranged from 20° to 23°C. Behavioral observations were usually made on anemones in their culture dishes. One hour before each experiment, the natural sea water was changed and the animals were placed on the microscope with the lights on to allow them time to adjust. For the behavioral interactions, both animals were allowed to become well expanded before they were moved into contact. The outcome was recorded with still and motion pictures using Nikon FTN (35 mm), Nizo (Super 8 mm), and Bolex (16 mm) cameras; with an Esterline Angus 190-MT event recorder; or by using a stop watch and hand counter with handwritten notes.

To provide more control over the eliciting stimuli, a 1-sec touch technique was used. For this technique an object, such as an excised tentacle or glass coverslip, was lightly touched to an anemone for about 1 sec every 30 sec. Successive contacts were made with the same area on the anemone, and unless otherwise stated, involved a group of four adjacent tentacles. This procedure was continued for 15 min, or until an acrorhagial response occurred. If acrorhagial expansion occurred within the 15 min, the contacts were continued until the rest of the response was elicited. As discussed below (Fig. 9), more than 97% of normal intractions occurred within 15 min. An acrorhagus was considered to be expanded when it enlarged to $\frac{1}{2}$ the length of adjacent tentacles.

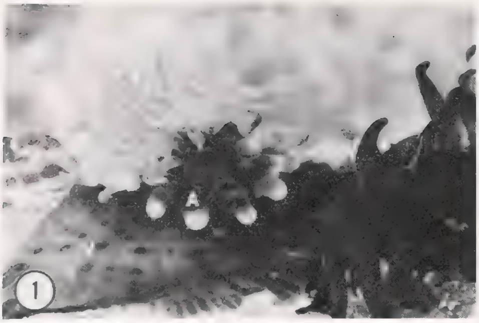
Excised tentacles of *C. gigantea* and *A. sargassensis* were used to investigate short term changes in the thresholds of acrorhagial expansion and application in *A. krebsi* with repeated target contacts. The threshold was defined as the number of touches, using the 1-sec touch technique with an excised tentacle, required to elicit the response from the specimen of *A. krebsi*. In this experiment, the threshold was determined, a period of time was allowed to elapse, and the threshold was determined again for up to 10 determinations. The time intervals used were 10 min for the *C. gigantea* stimulus and 15, 30, 60, and 120 min for the *A. sargassensis* stimuli.

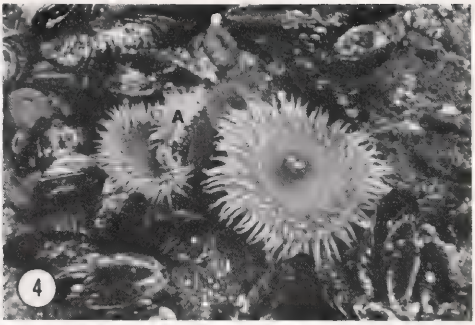
RESULTS

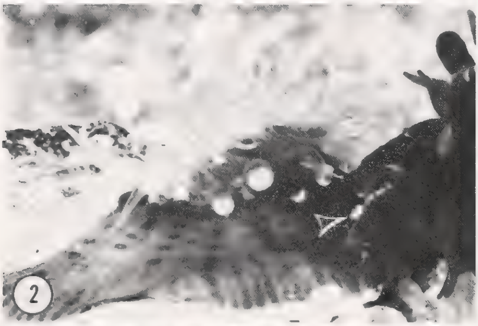
Behavioral alternatives to the acrorhagial response

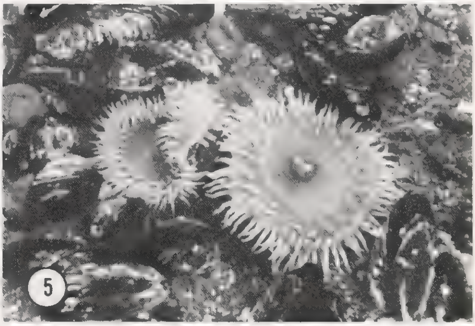
During anemone encounters, observable acrorhagial responses occurred only after physical contact between the anemones. Usually, the contact was initially made by the tentacles and resulted either in an interlacing of the tentacles of both animals with no change in behavior, or a rapid withdrawal of the tentacles of one or both animals. Generally all the tentacles, but often only the tentacles in the area of contact, were withdrawn. The anemones then re-extended the tentacles and the process was repeated until one of three events occurred: 1) after several tentacular withdrawals, the tentacles of the two anemones interlaced and each animal treated the other as an inert object; 2) one or both anemones avoided contact with the other individual; 3) the acrorhagial response was initiated by one or both anemones.

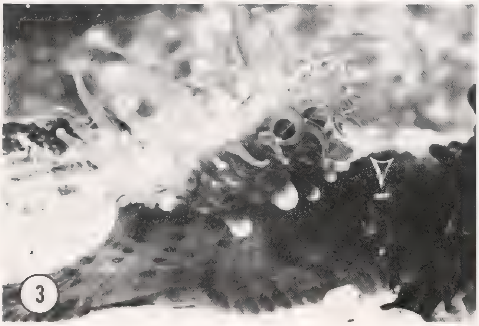
Anemones avoided contact with other anemones by bending the column away, only partially expanding the tentacles on the side of contact, using the pedal disc to











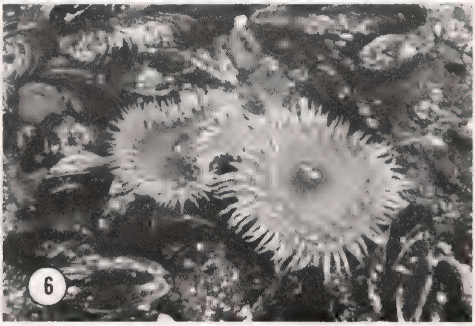


FIGURE 1. The specimen of *Anthopleura krebsi* is in the process of applying its expanded acrorhagi on the target, *A. sargassensis.* A—acrorhagi, V—verruca.

FIGURE 2. The specimen of *Anthopleura krebsi* has just withdrawn its acrorhagi from the target, *A. sargassensis.* The arrow points to an *A. krebsi* ectodermal peel adhering to the target. Note the corresponding "clear" area on the *A. krebsi* acrorhagus tip from which it originated.

FIGURE 3. Following *peel* (arrow) application by *Anthopleura krebsi* the target anemone, *A. sargassensis,* retracts the afflicted tentacle.

FIGURE 4. The specimen of *Anthopleura xanthogrammica* on the left has expanded its acrorhagi (A) adjacent to the *A. xanthogrammica* on the right.

FIGURE 5. The left specimen of *A. xanthogrammica* is in the process of applying its expanded acrorhagi to the right *A. xanthogrammica.* Note the capitulum is also expanded.

FIGURE 6. The left specimen of *A. xanthogrammica* is applying its expanded acrorhagi to the other specimen of *A. xanthogrammica.*

creep away, and releasing the pedal attachment to the substrate. Under the experimental conditions of this study, an unattached anemone remained in the same general position and contact with the other anemone was not avoided. However, in the field the wave surge or current in all *A. krebsi, B. cavernata* and *A. sar-*

gassensis habitats would quickly remove an unattached anemone. Moving away and releasing the hold on the substrate were by far the most common responses of specimens of *A. sargassensis* to other species of anemone and to the predatory nudibranch *Spurilla neapolitana*. *Bunodosoma cavernata* responded to *S. neapolitana* by releasing its hold on the substrate. It also withdrew its tentacles and greatly inflated its column. Inflating the column and floating free are two common anemone responses to nudibranch predation (Edmunds *et al.*, 1976).

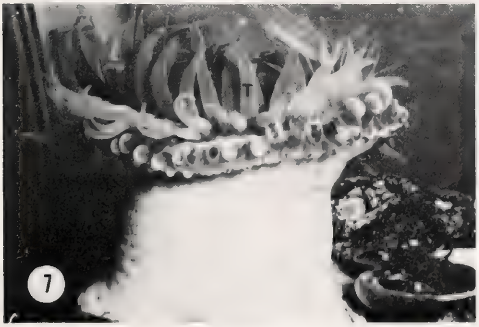
Another anemone response to encounters with other animals was predation. Such responses to other coelenterates were of particular interest. Although in some cases feeding behavior and the acrorhagial response were elicited by the same species (Table II), the two behaviors were mutually exclusive. One or the other occurred, but never feeding and the acrorhagial response during the same encounter.

The general acrorhagial response

The five general phases of the acrorhagial response in *A. krebsi, A. xanthogrammica* and *B. cavernata* are similar to those found in other actinians (Francis, 1973a, b; Bonnin, 1964): 1) excitation, contact and tentacular withdrawal; 2) acrorhagi expansion (Figs. 1 and 7); 3) application, with the acrorhagi directed to the area of stimulation (Figs. 2, 4, 5, and 8); 4) acrorhagial peeling, discharge of nematocysts and adherence of acrorhagial ectoderm to the target (Figs. 3, 6, and 5) recovery, return to normal posture. Cytological and ultrastructural examinations of the acrorhagus and events of the acrorhagial response are contained in separate reports in preparation.

Anthopleura krebsi *acrorhagial response*

The excitation period was defined as the time from first contact of the animals to the acrorhagial expansion. Its duration is variable, particularly depending on the species eliciting the response. *A. krebsi* intraspecific response excitation periods varied from 20 sec following a single contact to 25 min, with a mean of 4.9 min (Fig. 9). On the other hand, no responses were elicited by *Cassiopea* in less than 3 min. As in *A. equina* (Bonnin, 1964), *A. krebsi* tentacles in the region of acrorhagi expansion usually deflate as the acrorhagi expand. Many times, how-



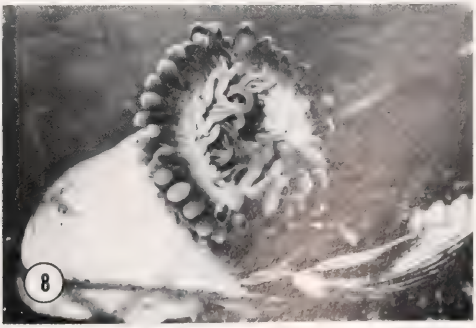


Figure 7. *Bunodosoma cavernata* acrorhagi (A) are starting to expand and the tentacles (T) are being drawn into the oral area. Peels were missing from some acrorhagi due to a previous response.

Figure 8. *Bunodosoma cavernata:* Note the tentacles held in the oral area and prominent acrorhagi around the oral disc during application behavior.

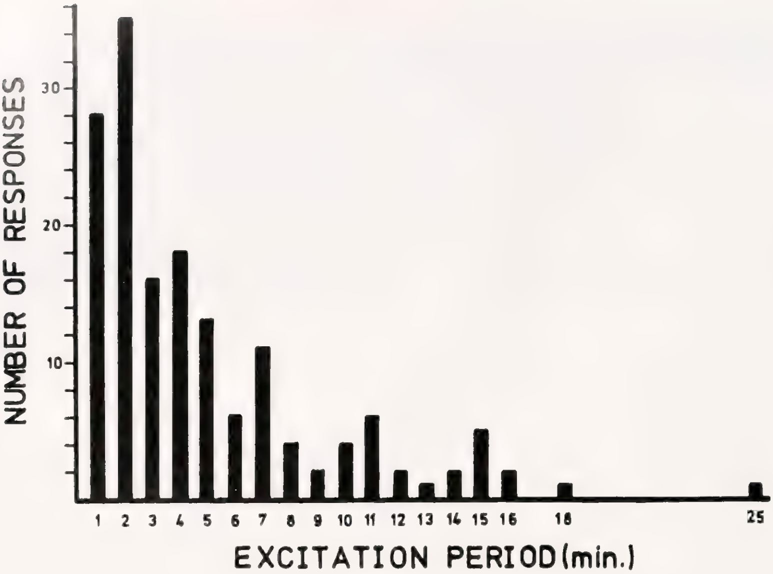


FIGURE 9. Duration of the excitation periods of 157 *A. krebsi* intraspecific acrorhagial responses. The excitation period was defined as the time from first contact of the animals to the expansion of an acrorhagus to one half the length of adjacent tentacles.

ever, there is no appreciable decrease in tentacle size and the fluid used to inflate the acrorhagi appears to come from the main coelenteron.

In most responses, application behavior immediately followed the acrorhagial expansion (Fig. 10). However, during some responses, several minutes elapsed between acrorhagial expansion and the onset of application behavior. Application behavior was never initiated during some acrorhagial responses, and in many of these instances, the tentacles and column were contracted so that the acrorhagi were prominent. Even without application, a touch of the eliciting animal to an expanded acrorhagus caused an acrorhagial peel.

As in *A. elegantissima* (Francis, 1976), the number of acrorhagi a specimen of *A. krebsi* possesses varies and is not strictly proportional to anemone size. Typically, as the application phase of the acrorhagial response progresses, more acrorhagi expand. The total number of expanded acrorhagi (usually 2–8) varies

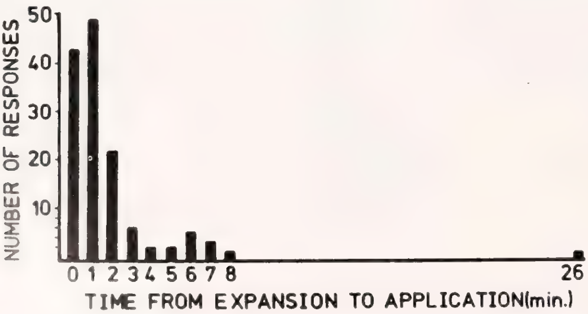


FIGURE 10. Times from acrorhagial expansion to the onset of application behavior during 134 *A. krebsi* intraspecific acrorhagial responses.

with the individual. Also, as the application progresses, the capitulum in the region of the expanded acrorhagi usually expands. Acrorhagial application is directed toward the area of stimulation. When the stimulus, either an excised tentacle or a whole anemone, was moved around a specimen of *A. krebsi*, the zone of acrorhagi expansion and direction of application followed the stimulus.

During application the expanded acrorhagi are pressed tightly against the target, in many instances for more than 30 sec. When an acrorhagus is withdrawn, a patch of the acrorhagus-tip ectoderm, the peel, remains on the target. As reported for *A. elegantissima* (Francis, 1973b) and *A. equina* (Bonnin, 1964), *A. krebsi* returns to a normal posture following the acrorhagial response. Typically, after removal of the target, the acrorhagi are applied to the former position of the target animal several times. After the application ceases, the acrorhagi usually return to their unexpanded state in approximately 10 min.

Anthopleura xanthogrammica *acrorhagial response*

Anthopleura xanthogrammica was previously reported to be the sole case of an acrorhagi-bearing anemone lacking an acrorhagial response (Francis, 1973b). However, *A. xanthogrammica* acrorhagial responses were observed under field conditions (Figs. 4–6). A pair of touching specimens of *A. xanthogrammica* in a small tidal pool were watched for approximately 15 min (also observed by L. Minasian and E. Conklin). When first seen, one of the anemones had expanded acrorhagi in the region adjacent to the other anemone and was in the midst of application behavior. During the observation period, the former target anemone also initiated an acrorhagial response. The response of the former aggressor then subsided. This second response continued until I left. Upon my return approximately 1 hr later, both anemones were contracted and there was no longer any contact. The *A. xanthogrammica* acrorhagial response consisted of acrorhagial expansion, application behavior, and peeling, as described for *A. equina* (Abel, 1954; Bonnin, 1964), *A. elegantissima* (Francis, 1973b), and *A. krebsi*.

Bunodosoma cavernata *acrorhagial response*

Bunodosoma cavernata has the previously described five phases in its acrorhagial response. However, *B. cavernata* usually maintains a different posture during the application behavior. Instead of expanding the capitulum and drawing back the tentacles in the region of acrorhagial expansion, *B. cavernata* contracts its tentacles into the oral area. This leaves the swollen acrorhagi as the sole protuberances on the upper column (Fig. 8). On other occasions, however, the *B. cavernata* application posture more closely resembles that of *A. krebsi*.

Anemonia sargassensis *acrorhagial response*

In laboratory studies, *Anemonia sargassensis* very seldom displayed an acrorhagial response. Usually the anemones moved away from contact with others. In the 10 observed *A. sargassensis* acrorhagial responses, the acrorhagi expanded, although generally not as much as in *B. cavernata* or *Anthopleura*. The tentacles were not retracted as much as those of *B. cavernata* and, in most cases, not even as much as the tentacles of *A. krebsi*. Application behavior was not observed in *A. sargassensis*. In all instances, when *A. krebsi* or *B. cavernata* contacted a turgid

A. sargassensis acrorhagi a peel occurred, but there was no directed application of a peel.

Effects of the peel on the target animal

Acrorhagial peels had three gross effects on both allogeneic and xenogeneic target animals: behavioral, mechanical, and necrotic. The first two effects will be discussed here. The peels of *A. krebsi*, *B. cavernata*, and *A. sargassensis* appeared to produce the same results, except the larger size of some *B. cavernata* peels magnified the effect. Anemones had three common behavioral responses to receiving a peel. Anemones receiving a peel on a tentacle, especially *A. sargassensis*, often contracted the tentacle (or tentacles) involved (Fig. 4). These tentacles remained contracted even when all the other tentacles were expanded. In some specimens of *A. sargassensis*, the affected tentacles remained contracted more than 24 hr after the application of a peel. After several peels, the anemones usually released their hold on the substrate and under field conditions would have been washed away. Upon receiving peels, specimens of *A. krebsi* commonly contracted, and using the pedal disc, moved away. On several occasions, large peels bound the tentacles or acrorhagi of a target anemone together, causing a mechanical impediment to their normal use. In three cases, 24 hr after an application the tentacles were still bound together.

Acrorhagial response specificity

To test the specificity of the acrorhagial response, various objects were applied with the 1-sec touch technique to *B. cavernata* tentacles. In five trials, glass coverslips, *Artemia*-extract-coated coverslips (which elicited nematocyst discharge from the tentacles), a stainless steel probe, and previously excised tentacles from the opposite side of the test anemone, all failed to elicit expansion or application behavior. Following each failure, an acrorhagial response was elicited from the specimens of *B. cavernata* by an excised *A. sargassensis* tentacle.

As with *A. krebsi* (Bigger, 1976), the specificity of acrorhagial peeling was tested in *B. cavernata* and *A. sargassensis*. An excised tentacle of *A. krebsi* was used to elicit an acrorhagial response from *B. cavernata;* then, following an acrorhagial peel, the excised tentacle was removed. A previously excised tentacle from the *B. cavernata,* a glass coverslip, and an *Artemia*-extract-coated coverslip were each touched ten times to expanded acrorhagi. In ten trials, they did not elicit a peel. The excised *A. krebsi* tentacles were then again touched to expanded acrorhagi and, in all instances, a peel resulted from the first contact. The same protocol was used in five trials with *A. sargassensis* and the same results were obtained.

Response threshold change

The acrorhagi expansion and acrorhagial application thresholds of *A. krebsi* were determined for repeated responses to see if either habituation or sensitization occurred. The response of *A. krebsi* to *C. gigantea* tentacles was tested at 10-min intervals with the 1-sec touch technique. The *A. krebsi* response to *A. sargassensis* tentacles was likewise tested for 15-, 30-, 60-, and 120-min intervals. In all cases (Fig. 11), there was a rapid decrease in threshold for the second set of stimulations. With 15-, 30-, 60-, and 120-min intervals, the threshold remained low. After 60 min of stimulation at 10-min intervals, some specimens of

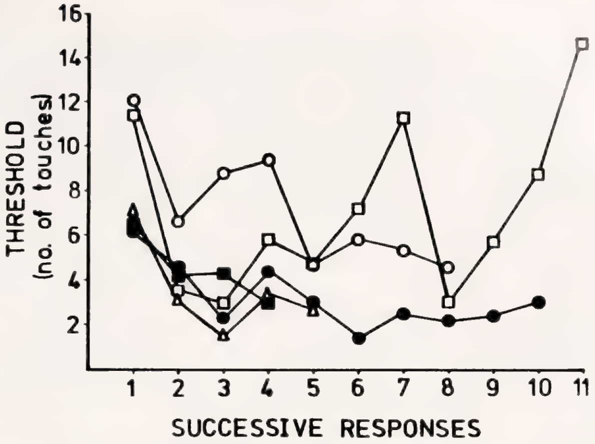


FIGURE 11. Changes in *A. krebsi* acrorhagial response with elicitation at intervals of 10–120 min. Using the 1-sec touch technique with excised *C. gigantea* (10 min) and *A. sargassensis* (15–120 min) tentacles, acrorhagial responses were elicited from *A. krebsi* at various time intervals. The threshold was defined as the number of touches required for acrorhagial expansion. The average thresholds of 10 individuals for each time interval are displayed in this graph. Open box = 10 min, closed circle = 15 min, open circle = 30 min, open triangle = 60 min, and closed box = 120 min.

A. krebsi would not respond. In most cases, the anemone would respond during the following stimulation periods. The time of these refractory periods varied among the anemones tested. Following the fifth stimulation period, some of the test anemones were in a refractory period and some were responding normally. This variability is responsible for the high values in the later part of the 10-min-interval curve in Figure 11.

In this study, the effect of size on the acrorhagial response was not specifically studied. Specimens of *Anthopleura krebsi* of approximately the same size were paired in most intraspecific interactions. However, in three cases groupmates of various size were tested against the same target animals. The groupmate/target size ratios for individuals in the three groups were: 1) 3 of 1 : 1, 1 of 2 : 3, and 2 of <1 : 2; 2) 2 of 1 : 1, 2 of 2 : 3, and 2 of <1 : 2; 3) 1 of 1 : 1 and 1 of 3 : 2. In these tests, the combination rather than size was the best correlate of which anemone was first to respond, *i.e.*, all individuals of a group performed the same with the same target animal. In the field, specimens of *A. krebsi* have been observed to initiate an acrorhagial response to larger nonresponding non-groupmates, but the prior history of pairs was unknown and controls were lacking. In *A. krebsi*/*B. cavernata* interactions, *A. krebsi* usually responded before the much larger *B. cavernata*; however, upon receiving *B. cavernata* peels (one application could cover a large portion of the *A. krebsi*) the *A. krebsi* always ceased its attack and contracted.

Interspecific acrorhagial responses

The response of *A. krebsi* to *Bunodosoma granulifera* was tested with the 1-sec touch technique using intact *B. granulifera* tentacles as the stimulus. An acrorhagial response, including peeling, was elicited from *A. krebsi* in all three trials. Each interspecific interaction between *B. cavernata* or *A. sargassensis* and a variety

TABLE I

The interspecific nature of the Bunodosoma cavernata *acrorhagial response. The number of 1-hr interactions between a* B. cavernata *and a test animal is given in parentheses. Key: + = the response in question was elicited; — = the response in question was not elicited.*

Test animal	*B. cavernata* acrorhagial expansion	*B. cavernata* application behavior	*B. cavernata* acrorhagial peel
Anthozoa			
Anthopleura krebsi	+(5)	+(5)	+(5)
Anemonia sargassensis	+(5)	+(5)	+(5)
Calliactis tricolor	+(2), −(1)	+(2), −(1)	+(2), −(1)
*Condylactis gigantea**	−(2)	−(2)	−(2)
Bunodosoma granulifera	+(2), −(1)	+(2), −(1)	+(2), −(1)
Hydrozoa			
Campanularia sp.	−(5)	−(5)	−(5)
Non-coelenterates			
Pagurus longicarpus (hermit crab)	−(5)	−(5)	−(5)
Pagurus pollicaris (hermit crab)	−(5)	−(5)	−(5)
Spurilla neapolitana (nudibranch)	−(5)	−(5)	−(5)

* Test animals were ingested by the specimen of *B. cavernata*.

of animals was observed for 1 hr to determine how extensively the acrorhagial response occurred in interspecific encounters (Tables I and II). The results followed the same pattern as in earlier reports on *A. equina* (Bonnin, 1964), *A. elegantissima* (Francis, 1973b) and *A. krebsi* (Bigger, 1976). Sea anemones usually elicited an acrorhagial response from *B. cavernata*, whereas hydroids and

TABLE II

The interspecific nature of the Anemonia sargassensis *acrorhagial response. The number of 1-hr interactions between a specimen of* A. sargassensis *and a test animal is given in parentheses. Key: + = the response in question was elicited; — = the response in question was not elicited.*

	A. sargassensis acrorhagial expansion	*A. sargassensis* application behavior	*A. sargassensis* acrorhagial peel
Anthozoa			
*Anthopleura krebsi**	+(2), −(3)	−(5)	+(2), −(3)
Bunodosoma cavernata	+(1), −(4)	−(5)	+(1), −(4)
Bunodosoma granulifera	−(3)	−(3)	−(3)
*Calliactis tricolor**	−(5)	−(5)	−(5)
*Bunodactis texaensis**	−(3)	−(3)	−(3)
Hydrozoa			
Campanularia sp.	−(5)	−(5)	−(5)
Non-coelenterates			
Pagurus longicarpus (hermit crab)	−(5)	−(5)	−(5)
Pagurus pollicaris (hermit crab)	−(5)	−(5)	−(5)
Spurilla neapolitana	−(5)	−(5)	−(5)

* Specimens of *A. sargassensis* usually avoided contact with the test animal (discussed in text).

non-coelenterates did not. The *A. sargassensis* used in this study tended to avoid contact rather than to employ the acrorhagial response against coelenterates and showed no acrorhagial response toward non-coelenterates.

Bigger (1976) reported the only observations of a non-anthozoan (scyphozoan) eliciting an acrorhagial response. *Anthopleura krebsi* acrorhagial responses were elicited during two of three encounters with *C. xamachana* scyphistomae. Surprisingly, the *Cassiopea* medusae in that study did not elicit an acrorhagial response from *A. krebsi*. The medusae and scyphistomae had been collected at different geographical locations and the species identification of the polyp was uncertain (later confirmed as *Cassiopea xamachana*).

To further examine those reported differences in *A. krebsi's* response to *Cassiopea xamachana* medusae and scyphistomae, 64 1-sec touch tests were performed with scyphistomae and 42 tests with medusae (Table III). To eliminate possible genetic differences, all of the polyps and medusae were from the same clone (raised from a single polyp). Approximately the same percentage of *C. xamachana* polyps and medusae elicited acrorhagial expansion from *A. krebsi*. The percentage of medusae eliciting the application behavior was about half that of the polyps. Most important, all of the polyps that elicited acrorhagial expansion elicited an acrorhagial peel. None of the medusae elicited a peel, even when touched 15–30 times to an expanded acrorhagus.

Intraspecific interactions in A. krebsi

In order to test an intraspecific competition model proposed for the acrorhagial response by Francis (1973b), as well as the more general competition models (Jackson and Buss, 1975; Connell, 1976), the interactions of seven groups of *A. krebsi* were examined in a series of 1-hr observations. The groups were from sites 60 cm to 250 m apart (separated by sand). The study groups consisted of five groups of the red color morph and two of the green. The groups were maintained in separate bowls and, in these observations, one anemone of a combination was moved (on its shell) to the bowl of the other group and placed so that reexpansion of its tentacles forced tentacle contact with one of the residents. The water was not changed prior to the interaction period. The combinations were planned as a 7 × 7 matrix with four replicates so that an anemone of each group was introduced into the bowl of every other group four times, and vice versa.

TABLE III

Response of Anthopleura krebsi *to contact with* Cassiopea xamachana. *Whole animals or, in some cases, excised medusae oral arms were applied to* A. krebsi *tentacles with the 1-sec touch technique. When acrorhagial expansion was elicited, the stimulus was applied to an expanded acrorhagus for at least 1 sec, 15 times.*

Cassiopea	Polyps	Medusae
No acrorhagial response	45 (70%)	28 (67%)
Acrorhagial response	19 (30%)	14 (33%)
Acrorhagial expansion	19 (30%)	14 (33%)
Application behavior	19 (30%)	7 (17%)
Peel Produced	19 (30%)	0
Total tested:	64	42

Table IV

Relative aggressiveness of seven A. krebsi *groups. As explained in detail in the text, each block of the matrix represents the results of eight 1-hr interactions of that combination. The set of interactions for each group pairing was scored for each group: moving away—1, contracting—2, receiving peels but remaining in place—3, remaining in place while the other anemone contracts or moves away—4, initiating the acrorhagial response and placing peels on the other anemone—5, no response during any of the interaction—NR. Total score for the horizontal group was subtracted from the diagonal group to obtain a measure of the relative aggressiveness of the groups; i.e., 0 –both groups of a combination were equally aggressive, "−" score –the horizontal group was the more aggressive of the pairing, and "+" score—the diagonal group was the more aggressive of the pairing.*

	8cl	4dl	5ea	62	6e	3e	2e
8cl	NR	−16	−12	−6	−2	0	+14
4dl		NR	−10	−9	−8	−4*	0
5ea			NR	NR	NR	NR	NR
62				NR	NR	NR	NR
6e					NR	NR	NR
3e						NR	NR
2e							NR

* Score based on only four interactions.

One group died of causes unrelated to the study toward the end of this investigation, leaving one combination and its reciprocal with four observations.

An anemone of each group was also removed and reintroduced to its groupmates (4 ×). No groupmates responded. The red groups never responded to another red group but did have acrorhagial responses to both green groups. The green groups directed acrorhagial responses toward each other and most red groups. Some intergroup combinations consistently resulted in the same group initiating an acrorhagial response, but in others the initiating group varied. Distance between the paired groups in the field did not seem to be an indicator of the outcomes of those interactions. In 25% of the interactions involving an acrorhagial response, both anemones initiated a response.

The outcome of each interaction was scored in terms of aggressiveness for each member of the anemone pairs (Table IV). For each combination of two groups, one group's score was subtracted from the other group's score and divided by the number of interactions for each combination to give a measure of the relative aggressiveness of the two groups under those conditions.

Whether anemones were introduced or were residents in the interactions did not significantly (Wilcoxan signed-rank test) affect the outcome. Therefore, all interactions (8) of each group combination are combined and given in Table IV as a relative measure of the aggressiveness of the responsive groups.

Discussion

The discovery of such a complex behavior as the acrorhagial response in the morphologically relatively simple actinians has raised a number of questions. What is the behavioral nature of the response? What animals will elicit the response? Do factors such as prior experience, relative size, residence, and genotype of the interacting anemones affect the outcome? What is the functional significance of the response? Is the acrorhagial response related to other coelenterate self not-self recognition systems or to an immune system?

Despite an earlier report to the contrary (Francis, 1973b) and a single observation under unusual circumstances (Lindberg, 1976) the current study presents an observation of *A. xanthogrammica* displaying an acrorhagial response under field conditions, so that all anemones with acrorhagi that have been examined are known to display a similar acrorhagial response (Abel, 1954; Bonnin, 1964; Francis, 1973b; Bigger, 1976). As pointed out by Francis (1973b) the acrorhagial response meets the general definition of an aggressive behavior. Some definitions (*e.g.*, Hinde, 1970) require an aggressive behavior to be directed towards the other individual, a criterion met by the application component of the acrorhagial behavior of all species examined except *A. sargassensis*. Even in that instance, one can make a case for the directed nature of the *A. sargassensis* acrorhagial response because the specificity of the peeling insures a directed nature; *i.e.*, the response can only go to completion upon contact with the proper animal. Of special interest is the fact that anemones will respond to the same species as food or as an acrorhagial target, but at different times. Although there is a fine line between predation and aggression, in some cases, (*i.e.*, in corals, Lang, 1971 and 1973) the same phenomenon might be considered as both. Because predation and the acrorhagial response are mutually exclusive, one need only consider the acrorhagial response in terms of aggression.

Bonnin (1964) demonstrated that induction of an acrorhagial response in *A. equina* caused a lowering of the threshold for subsequent acrorhagial responses elicited at 10-min intervals, and that by the sixth acrorhagial response specimens of *A. equina* became unresponsive to further stimulation. In the present study, a similar threshold lowering and elevation was observed when the *A. krebsi* acrorhagial response was elicited at 10-min intervals. However, not all specimens of *A. krebsi* became totally refractory and at longer intervals between response elicitation (15+ min), the *A. krebsi* acrorhagial response threshold remained low (Fig. 11). In *A. krebsi*, prior experience influenced later responses over periods as long as 2 hr; this should be considered in experimental design.

Acrorhagial responses have not been elicited by non-coelenterates in the five anemone species that have been examined: *A. equina* (Bonnin, 1964), *A. elegantissima* (Francis, 1973b), *A. krebsi* (Bigger, 1976), *A. sargassensis* and *B. cavernata* (this study). Therefore, these sea anemones must compete with non-coelenterates for resources in some other fashion. To date (Abel, 1954; Bonnin, 1964; Francis, 1973b; Bigger, 1976), acrorhagial responses have only been elicited by some sessile anthozoans or *C. xamachana*. Past consideration of the role of the acrorhagial response has emphasized intraspecific interactions on the grounds that those were much stronger than the interspecific acrorhagial responses (Francis, 1973b). The highly predictable, full acrorhagial responses of either *A. krebsi* or *B. cavernata* to *A. sargassensis* indicate that acrorhagial responses could be effectively employed against some other anthozoans, but the lack of pertinent field data limits a full assessment of the interspecific role of the acrorhagial response.

This study amplifies the preliminary report (Bigger, 1976) of a difference in the response of *A. krebsi* to polyps and medusae of the scyphozoan *C. xamachana*. With twice the effectiveness of the medusa in soliciting acrorhagial application, the polyp appears either to have a qualitatively more effective application-eliciting factor or to contain more of the eliciting factor. In the light of Bonnin's suggestion (1964) that the nematocysts of the target animal constitute the eliciting factor, it should be noted that the nematocysts of polyp tentacles and medusa mouth fronds (an area that touched the anemones) are morphologically the same and appear to be present in roughly equivalent numbers (Mariscal and Bigger, 1976; unpublished observations). Nematocyst toxins from *Cassiopea* medusae and scyphistomae have not been compared. For the most important measure of the responses, peel elicitation, 30% of the polyps, but never the medusae, elicited peels. These results point out the separation between the acrorhagial response components (acrorhagial expansion, application behavior, and ectodermal peeling; see Bigger, 1976) and suggest three possibilities: 1) Each component may require a different eliciting factor (or combination of factors). The medusae and polyps would contain the expansion factor but differ in their complement of other factors. 2) The receptors for the three components may have different thresholds for the same factor. The medusae and polyps would have enough of the eliciting factor to exceed the acrorhagial expansion threshold but would be quantitatively differentiated by the receptors of the other two components. 3) The discrimination would be based on some combination of the first two. At this time there are not enough data to suggest one possibility more than another.

Francis (1973b) proposed that the acrorhagial response primarily functioned in intraspecific competition for space. Central to Francis' hypothesis is the concept that anemones distinguish clonemates from all other conspecifics, a concept derived from observations of *A. elegantissima* acrorhagial responses being elicited by all non-groupmates ("non-clonemates"). This study shows that not all *A. krebsi* non-groupmates elicit a response. In fact, some specimens of *A. krebsi* with different color morphs, one of Francis' criteria for non-clonemates, did not respond to each other. Therefore, one cannot consider the acrorhagial response to be solely a case of an "individual" (clone) recognizing all other conspecifics as not-self and competing with them for available space. One must examine this as a case of related individuals (individual = clone) competing for space. These related individuals share alleles at some loci, which may include those determining surface molecules that participate in the recognition events of the acrorhagial response. The difference between the acrorhagial interaction of all *A. elegantissima* groups (Francis, 1973b) and the intergroup compatibility of some *A. krebsi* groups could reflect a true species difference or, alternatively, a more homogeneous gene pool in the specimens of *A. krebsi* sampled, such as the extremely limited or homogeneous gene pool in the Maine population of the sea anemone *Haliplanella luciae* (Schick, 1976). The data of Francis (1973b) and this study demonstrating the wide number of conspecific groups recognized by *A. elegantissima* and *A. krebsi* and the variability of the *A. krebsi* acrorhagial responses suggest multiple alleles coding for acrorhagial recognition and perhaps many different loci, *i.e.*, a complex polygenic phenomenon such as the mammalian histocompatibility system.

There is also a major genetic influence in other coelenterate self/not-self recognition systems, *e.g.*, overgrowth in hydroids (Ivker, 1972) and histoincompatibility in corals (Hildemann *et al.*, 1977) and gorgonians (Theodor, 1970). Thus reports of various factors, *e.g.*, size (Brace and Pavey, 1978), controlling the initiation of an acrorhagial response must be viewed with caution unless the genetic variable is controlled. Because there are no inbred strains, this is difficult with a solely sexually reproducing anemone. Asexually reproducing anemones such as *A. elegantissima* and *A. krebsi*, on the other hand, present the investigator with a large number of genetically identical anemones which allow reproducible group combinations under a variable experimental condition. Brace and Pavey (1978) reported a size hierarchy in the intitation of the acrorhagial response of *A. equina* (a solely sexually reproducing anemone), the larger anemones being first to respond. However, in approximately one third of the interactions of their study the smaller anemone was faster or as fast to initiate an attack. This indicates that other factors should be considered. Very limited evidence concerning the influence of size on the initiation of acrorhagial responses in *A. krebsi* suggests that size plays at most a subordinate role to the particular group combination ("histoincompatibility differences") in that species. The current study also suggests that residence in an area does not influence the outcome of *A. krebsi* interactions. Because the test anemones of this study were moved while still attached to their shells, the experimental design only allowed residence to be considered in terms of the anemone's surroundings and not on the basis of pedal attachment. Ottaway (1978), in his recent field observations of *Actinia tenebrosa*, noted "the successful aggressor was almost invariably the 'defender,' the anemone that had been stationary at the time of contact." Therefore, although general surroundings may not significantly influence the acrorhagial response, movement or long-term attachment may affect outcome.

If the acrorhagial response functions in competition for space, as proposed by Francis (1973a and b, 1976), one needs to explain coexistence of competitive groups. Several models for invertebrate competitive or aggressive interactions have been used to discuss interspecific situations (*e.g.*, Lang, 1973; Jackson and Buss, 1975; Connell, 1976). A hierarchy of aggression among corals has been reported by Lang (1971 and 1973). Connell (1976) suggested that such linear hierarchies are inherently unstable and that the intervention of an outside force that selectively acted against the higher ranked members of the hierachy could explain the concurrent existence of all the groups. Jackson and Buss (1975) and Buss (1976) proposed an interaction model of "competitive networks" rather than a linear dominance, *i.e.*, A > B > C > A, etc. Brace and Pavey (1978) reported such a "ring" situation in *A. equina* acrorhagial interactions and Table IV of the present study reveals one such network in *A. krebsi* interactions. However, contrary to the results of Brace and Pavey (1978) with *A. equina*, Table IV of this study indicates a high degree of variability in the outcomes of interactions between certain combinations of *A. krebsi*. Connell (1976) found the same variable outcomes in tissue destruction and overgrowth among the corals he studied. Buss (1976) states that such competitive networks function by increasing the time required for a dominant to be established and thereby reduce the magnitude of a disturbance required to maintain diversity. Rather than the acrorhagial response being viewed in a limited sense as only the mechanism whereby a clone can capture territory from conspecific competitors, the acrorhagial response can be viewed as one of a set of ecological factors possibly maintaining a heterogeneous gene pool and, through indirect interactions with other ecological factors, causing an optimal utilization of available space.

Hildemann and his associates (1975) specifically included the acrorhagial response when they categorized what they felt were four levels of "immunoreactivity" in coelenterates. In discussing the acrorhagial response, they recognized that a response elicited within minutes after first contact could represent non-immunological recognition but went on to suggest that because the anemones live in a crowded habitat, prior sensitization was not ruled out. Bigger (1976) reported the rapid elicitation of acrorhagial responses from *A. krebsi* by several allopatric species, including *Condylactis gigantea*, *C. xamachana*, and *Ceriantheopsis americanus*. A possible explanation, consistent with an immunological mechanism, for the rapid response to allopatric species would be that those allopatric target animals possessed a set of surface molecules so similar to those of previously encountered target animals that the two sets of molecules were perceived as the same. However, the concept of prior sensitization as a basis for the rapidity of the acrohagial response must be viewed with certain reservations.

More recently (Hildemann *et al.*, 1979), it has been suggested that three mimimal criteria must be met for a phenomenon to be considered immunologic: cytotoxic or antagonistic reactions, selective or specific reactivity, and inducible memory or selectively altered reactivity on secondary contact. Thus, while self/not-self recognition is certainly the cornerstone of immunology, not all self/not-self phenomena are immunologic in nature. Reactions among coelenterates involving self/not-self recognition include various cellular and behavioral phenomena in hydroids (Kato *et al.*, 1967; Ivker, 1972), gorgonians (Theodor, 1970; Bigger and Runyan, 1979), corals (Lang, 1971; Hildemann *et al.*, 1977), and sea anemones (Abel, 1954; Purcell, 1977). While all the above coelenterate responses might be considered to meet the first two criteria for an immune response, experiments examining the third criterion have been performed only with corals. Although Hildemann *et al.* (1977) demonstrated the characteristics of an immune system in corals, little is known about the recognition mechanisms, receptors, or molecular pathways involved, nor in some cases the effector cell types in the above mentioned coelenterate reactions. Until such information is acquired, suggestions of a common underlying recognition mechanism remain speculative. That the acrorhagial response utilizes a behavioral effector component does not preclude the use of a recognition system similar to that of other coelenterate self/not-self or immunological phenomena. However, the nature of the recognition along with many other questions about the functioning of the effector side of the acrorhagial response must await future investigations.

Dr. R. N. Mariscal's advice during this study and his critical reading of a preliminary draft of this manuscript were greatly appreciated. Thanks are also due Dr. W. H. Hildemann for critically reading this manuscript and offering valuable suggestions, Dr. M. J. Greenberg for suggestions, Dr. W. Herrnkind and the FSU Psychobiology Program for the loan of equipment, Steve White for statistical assistance, and Lois Bigger for translations. Some financial support for this study was provided by NSF Grant #DEB 77–22148 to Dr. R. N. Mariscal. Portions of this paper were submitted to Florida State University in theses in partial fulfillment of the requirements for the degrees of M.S. and Ph.D. This is contribution number 143, Tallahassee, Sopchoppy, and Gulf Coast Marine Biological Association.

Summary

The acrorhagial responses of four sea anemones, *Anthopleura krebsi*, *Bunodosoma cavernata*, *Anemonia sargassensis*, and *Anthopleura xanthogrammica*, are described. All four acrorhagial responses can be considered forms of aggression. The acrorhagial response is only one of several responses of sea anemones to contact with other animals; others include several methods of avoidance and feeding.

Prior experience can influence the acrorhagial response. In *A. krebsi*, the effect of a prior encounter on the excitation threshold can be seen for at least 2 hr.

Interspecific behavioral interactions were examined in *A. krebsi*, *B. cavernata*, and *A. sargassensis*. With one exception, acrorhagial responses were only elicited by contact with some anthozoans. The exception is that some *A. krebsi* respond to the scyphistomae of the scyphozoan *Cassiopea xamachana*. Some *C. xamachana* medusae from the same clone also elicited acrorhagial expansion and application behavior but never acrorhagial peeling.

Intraspecific interactions were examined in *A. krebsi*. Clonemates and groupmates never elicited acrorhagial responses from one another. Some non-groupmates, including different-colored groups, did not respond to one another and in some other group combinations the interactive outcome was variable. It is suggested the acrorhagial response involves multiple alleles and perhaps involvement of different loci coding for cell-surface recognition molecules. Several competition models were examined for these intraspecific interactions. An intergroup linear hierarchy was not found.

The acrorhagial response is certainly an example of self/not-self recognition. This response has exquisite specificity and leads to cytotoxic effects. It cannot at this time be considered immunological.

LITERATURE CITED

Abel, E. F., 1954. Ein Beitrag zur Giftwirkung der Aktinien und Funktion der Randsäckchen. *Zoologischer Anzeiger*, **153**: 259–268.

Bigelow, R. P., 1900. The anatomy and development of *Cassiopea xamachana*. *Boston Soc. Natur. Hist. Mem.*, **5**: 191–236.

Bigger, C. H., 1976. The acrorhagial response in *Anthopleura krebsi*: intraspecific and interspecific recognition. Pages 127–136 in G. O. Mackie, Ed., *Coelenterate ecology and behavior*. Plenum Press, New York.

Bigger, C. H., and R. Runyan, 1979. An *in situ* demonstration of self-recognition in gorgonians. *Develop. Compar. Immunol.*, **3**: 591–597.

Bonnin, J. P., 1964. Recherches sur la "reaction d'agression" et sur le fonctionnement der acrorrhages d'*Actinia equina* L. *Bull. Biol.*, **98**: 225–250.

Brace, R. C., and J. Pavey, 1978. Size-dependent dominance hierarchy in the anemone *Actinia equina*. *Nature*, **273**: 752–753.

Buss, L. W., 1976. Better living through chemistry: the relationship between allelochemical interactions and competitive networks. Pages 315–327 in F. W. Harrison and R. R. Cowden, Eds., *Aspects of sponge biology*. Academic Press, New York.

Carlgren, O., and J. W. Hedgpeth, 1952. Actiniaria, Zoantharia and Ceriantharia from shallow water in the Northwestern Gulf of Mexico. *Tex. Univ. Inst. Mar. Sci. Publ.*, **2(2)**: 141–172.

Connell, J. H., 1976. Competitive interactions and the species diversity of corals. Pages 51–58 in G. O. Mackie, Ed., *Coelenterate ecology and behavior*. Plenum Press, New York.

Dixon, G. Y., and A. F. Dixon, 1891. Report on marine invertebrate fauna near Dublin. *Proc. Royal Irish Acad.*, (3)2: 19–33.

Duncan, P. M., 1874. On the nervous system of *Actinia, Part I*. *Proc. Royal Soc. Lond.*, **22**: 263–276.

Edmunds, M., G. W. Potts, R. C. Swinfen, and V. L. Waters, 1976. Defensive behavior of sea anemones in response to predation by the opisthobranch mollusc *Aeolidia papillosa* (L.). *J. Mar. Biol. Assoc. U.K.*, **56**: 65–83.

Francis, L., 1973a. Clone specific segregation in the sea anemone *Anthopleura elegantissima*. *Biol. Bull.*, **144**: 64–72.

Francis, L., 1973b. Intraspecific aggression and its effect on the distribution of *Anthopleura elegantissima* and some related sea anemones. *Biol. Bull.*, **144**: 73–92.

Francis, L., 1976. Social organization within clones of the sea anemone *Anthopleura elegantissima*. *Biol. Bull.*, **150**: 361–376.

Gosse, P. H., 1860. *A history of British sea-anemones and corals.* Van Voorst, London, 362 pp.

Hildemann, W. H., D. S. Linthicum, and D. C. Vann, 1975. Immunoincompatibility reactions in corals (Coelenterata). Pages 105–114 in W. H. Hildemann and A. A. Benedict, Eds., *Immunologic phylogeny.* Plenum Press, New York.

Hildemann, W. H., R. L. Raison, G. Cheung, C. J. Hull, L. Akaka, and J. Okamoto, 1977. Immunlogical specificity and memory in a scleractinian coral. *Nature,* **270**: 219–223.

Hildemann, W. H., C. H. Bigger, and I. S. Johnston, 1979. Histoincompatibility reactions and allogeneic polymorphism among invertebrates. *Transplant. Proc.,* **11**: 1136–1141.

Hinde, R. A., 1970. *Animal behavior* (2nd edition). McGraw-Hill, New York, 876 pp.

Hollard, H., 1851. Monographie anatomique due genre Actinia de Linné, consideréré comme type du groupe général des Polypes Zoanthaires (d'après les *Act. senilis* et *equina*). *Ann. Sci. Nat. Zool. Biol. Anim.* **(3)15**: 257–291.

Ivker, F. B., 1972. A hierarchy of histo-incompatibility in *Hydractinia echinata. Biol. Bull.,* **143**: 162–174.

Jackson, J. B. C., and L. Buss, 1975. Allelopathy and spatial competition among coral reef invertebrates. *Proc. Nat. Acad. Sci., U.S.A.,* **72**: 5160–5163.

Kato, H., E. Hirai, and Y. Kakinuma, 1967. Experiments on the coaction among hydrozoan species in the colony formation. *Sci. Rep. Tohoku Univ. Ser. IV (Biol.),* **33**: 359–373.

Korotneff, M. de, 1876. Organes des sens des Actinies. *Arch. Zool. Exp. Gen.* **5**: 203–208.

Lang, J., 1971. Interspecific aggression by scleractinian corals. 1. The rediscovery of *Scolymia cubensis* (Milne Edwards and Haime). *Bull. Mar. Sci.,* **21**: 952–959.

Lang, J., 1973. Interspecific aggression by scleractinian corals. 2. Why the race is not only to the swift. *Bull. Mar. Sci.,* **23**: 260–279.

Lindberg, W. J., 1976. Starvation behavior of the sea anemones, *Anthopleura xanthogrammica* and *Metridium senile. The Biologist,* **58**: 81–88.

Mariscal, R. N., and C. H. Bigger, 1976. A comparison of putative sensory receptors associated with nematocysts in an anthozoan and a scyphozoan. Pages 559–568 in G. O. Mackie, Ed., *Coelenterate ecology and behavior.* Plenum Publishing Corporation, New York.

Ottaway, J. R., 1978. Population ecology of the intertidal anemone *Actinia tenebrosa.* I. Pedal locomotion and intraspecific aggression. *Aust. J. Mar. Freshw. Res.,* **29**: 787–802.

Pantin, C. F. A., 1952. The elementary nervous system. *Proc. R. Soc. Lond. B. Biol. Sci.,* **140**: 147–168.

Purcell, J. E., 1977. Aggressive function and induced development of catch tentacles in the sea anemone *Metridium senile* (Coelenterate, Actinaria). *Biol. Bull.,* **153**: 355–368.

Rapp, W., 1829. *Über die Polypen in Allgemeinen und die Aktinien im Besonderen,* Verlage des Grofsherzogl. Sächs. privileg. Landes-Industrie-Comptoirs, Weimar, 62 pp.

Ross, D. M., 1974. Behavior patterns in associations and interactions with other animals. Pages 281–312 in L. Muscatine and H. M. Lenhoff, Eds., *Coelenterate Biology: Reviews and New Perspectives.* Academic Press, New York.

Schick, J. M., 1976. Ecological physiology and genetics of the colonizing actinian *Haliplanella luciae.* Pages 137–146 in G. O. Mackie, Ed., *Coelenterate ecology and behavior.* Plenum Press, New York.

Theodor, J., 1970. Distinction between "self" and "not-self" in lower invertebrates. *Nature,* **227**: 690–692.

Walton, C. L., 1910. On some colour variations and adaptations in Actiniae. *J. Mar. Biol. Assoc. U.K.,* **9**: 228–235.

Williams, R. B., 1978. Some recent observations on the acrorhagi of sea anemones. *J. Mar. Biol. Assoc. U.K.,* **58**: 787–788.

Reference: *Biol. Bull.* **159**: 135–147. (August, 1980)

THE IMPLICATION OF CARBONIC ANHYDRASE IN THE PHYSIOLOGICAL MECHANISM OF PENETRATION OF CARBONATE SUBSTRATA BY THE MARINE BURROWING SPONGE *CLIONA CELATA* (DEMOSPONGIAE)

WALTER I. HATCH

Department of Biology, Southeastern Massachusetts University,
North Dartmouth, Massachusetts 02747

Marine sponges of the family Clionidae (Gray, 1867) are structurally and functionally similar to other members of the class Demospongiae, phylum Porifera (Vosmaer, 1933–1935; Hyman, 1940). They are, however, unique among the sponges in that each species in the family has the ability to excavate a habitation, in the form of dendritic or anastomosing galleries, in a variety of calcareous substrata (Hancock, 1849; Leidy, 1889).

In clionid sponges most of the tissues lie completely enclosed within the calcareous substratum, with the exception of numerous papillae which extend to the surface through holes in the substratum. Several ostial pores open into each incurrent papilla, giving them the appearnce of a sieve plate, while each excurrent papilla terminates in a single large osculum. Both types of papillae are strongly contractile and enable the sponge to insulate itself from unfavorable environmental conditions (Emson, 1966).

As the sponge grows, the galleries are enlarged and new channels established by the removal of numerous small chips, which are transported to the surface through the excurrent canal system in a manner analogous to a mining operation (Nassonow, 1883; Topsent, 1888; Cobb, 1969, 1971).

In many clionids the galleries remain discrete and the sponge does not completely destroy the substratum. In *Cliona celata,* however, the sponge tissue spreads from the base of the papillae to cover the surface of the substratum, continually removing carbonate until the galleries fuse and the substratum is completely eliminated, leaving an unenclosed free-living sponge. Although this is a continuous process, for convenience it has been divided into three stages: alpha stage while the sponge is completely enclosed, grading into beta stage as the sponge extends across the surface, and finally gamma stage when the substratum has been completely destroyed (Vosmaer, 1933–1935).

The nature of the mechanism employed by the Clionidae to excavate calcareous substrata has been the subject of investigation, speculation, and controversy since 1826, when Osler first described a sponge-like organism within the valves of oysters (Osler, 1826). Three major hypotheses on the mode of penetration have been presented: an extensive chemical dissolution of the substratum, usually assumed to be the action of some acidic etching agent; an exclusively mechanical removal of the substratum, and a chemomechanical mechanism in which the substratum is chemically softened or loosened and subsequently mechanically removed. The most tenable mechanism is the combination of a localized chemical dissolution coupled with mechanical dislodging of fragments of the substratum and their subsequent transport out of the sponge galleries.

It seems evident from the manner in which chips are freed from the substratum (Cobb, 1969, 1971; Rutzler and Reiger, 1973) and from the fact that calcium carbonate is the primary mineral excavated by burrowing sponges, that the mechanism of excavation involves a localized modification of the calcium carbonate solubility equilibrium. Little progress has been made to date in the identification of an etching agent with this capability.

The potential difficulties of investigating penetration mechanisms that function at the cellular level suggest that a direct analysis of the clionid etching agent is not feasible at this time. This investigation was, therefore, undertaken not to demonstrate the presence of an acidic etching agent in *Cliona celata,* but rather to demonstrate the physiological capability of the sponge to produce such an agent or otherwise alter ion concentration products, thus affecting solubility.

The participation of the enzyme carbonic anhydrase in shifting solubility equilibria, resulting in the deposition of calcium carbonate by invertebrates, is well established (Goreau and Hartman, 1963; Heatfield, 1970; Istin and Girard, 1970). Carbonic anhydrase has been implicated in the dissolution of carbonate as well. The enzyme has been found in the accessory boring organ of shell-boring muricid gastropods (Chétail *et al.*, 1968; Smarsh *et al.*, 1969) and its activity has been related to the process of excavating calcium carbonate substrata by acrothoracican cirripeds (Turquier, 1968) and muricid gastropods (Chétail and Fournié, 1969; Carriker and Chauncey, 1974; Rosenberg *et al.,* 1968).

Thus, there is ample evidence that carbonic anhydrase operates in the dissolution and deposition of calcium carbonate in a variety of systems. The purpose of this investigation is to demonstrate this enzyme in actively excavating *Cliona celata* Grant, and its possible involvement in penetration of calcium carbonate by the sponge.

MATERIALS AND METHODS

Assay for clionid carbonic anhydrase

Carbonic anhydrase was assayed by an electrometric method (Wilbur and Anderson, 1948; Davis, 1963; Carter *et al.,* 1969; Carter, 1972).

Buffer preparation. Tris buffer (0.025 M, pH 8.8 at 0°C) was prepared by dilution of a 0.25 M stock solution immediately before use. The dilute buffer was protected from CO_2 contamination.

Substrate preparation. A saturated solution was prepared by bubbling pure CO_2 through deionized glass-distilled water, at 0°C, for at least 1 hr prior to use. The resulting substrate solution was 0.076 M CO_2, resulting in a final substrate concentration of 29.42 mM when a 2.50-ml aliquot was added to the 6-ml reaction mixture.

Enzyme preparation. Alpha-, beta-, and gamma-form specimens of *Cliona celata* from the Northwest Gutter of Hadley's Harbor, Naushon Island, Massachusetts, were maintained in running sea water. The sponges were identified by spicule examination (Old, 1941). Sponge tissue was obtained by drilling a 10-mm hole in the apex of shells in which alpha- and beta-form sponges were burrowing or by removing a plug of tissue from gamma-form sponges. This material was freed of shell fragments and sponge tissue by treatment with warm concentrated nitric acid, rinsed twice with distilled water and once with 95% ethanol, and spread on a slide for microscopic examination.

In preparation for enzyme extraction, living sponges were cleaned of epibiota by scraping and scrubbing briefly with a bottle brush and tap water. A sponge cell suspension was produced by manually expressing excess water, fragmenting with a hammer when necessary, and forcing the fragmented sponge tissue through a 2-mm stainless-steel sieve to remove inorganic inclusions and shell debris. Enzyme solutions were prepared by disrupting the cell suspension with a Waring blender. After dialysis (24 hr, distilled water at 0°C), enzyme solutions were diluted to 1–2 mg total protein/ml.

In order to eliminate the effects of non-catalytic protein buffering (Addink, 1971), controls were run in the presence of 10^{-3} M acetazolamide resulting in complete inhibition of enzyme activity. Inhibited enzyme solutions were prepared by adding 3.3 mg sodium acetazolamide to a 2.5 ml aliquot of dilute enzyme solution and titrating back to the pH of the uninhibited enzyme solution (7.25) with HCl.

Assay apparatus and procedure. The enzyme assay apparatus differed from that of Carter *et al.* (1969) in that the reaction vessel, delivery syringes, and reservoirs for buffer and substrate storage were all housed in a water bath thermostatically regulated to 0 ± 0.1°C. In addition, provision was made for transferring substrate solutions under positive pressure to avoid degassing and the resulting fluctuations in substrate concentration. A magnetic stirring device insured rapid mixing.

In use, 2.5 ml of buffer was transferred from delivery syringe to reaction vessel and 1.0 ml of either enzyme or control solution added. After 30–60 sec for electrode equilibration, 2.5 ml of substrate solution was injected and a chart recorder started simultaneously. The reaction was allowed to proceed to equilibrium before the reaction vessel was drained and flushed for the next determination.

The initial rate of catalyzed and control reactions was determined by fitting a tangent to the $[H^+]$-time curve at pH 8.70. From these tangents Δ pH/min could be determined and converted to mM H^+/min from a buffer-enzyme calibration curve. The enzymatic rate was determined by substracting mM H^+/min produced in control reactions from that produced in enzyme catalyzed reactions. The mean of triplicate runs was recorded as enzyme activity.

Intracellular localization of clionid carbonic anhydrase

Clionid cell suspensions were disrupted in 0.025 M sucrose at 0°C by alternating 30 sec of grinding with 5 min of cooling (4 ×) in a Waring blender. The resulting homogenate was centrifuged (10 min, 500 × *g*) to remove inorganic inclusions and unbroken cells. The supernatant was recentrifuged (10 min, 700 × *g*) over a layer of 0.34 M sucrose to sediment nuclei. The supernatant was again collected and recentrifuged (10 min, 21,000 × *g*) to sediment mitochondria-like particles. Each pellet was resuspended in distilled water and, along with the supernatant dialyzed against stirred distilled water for 24 hr at 0°C. Each retentate was then assayed for carbonic anhydrase activity.

An aliquot of the supernatant (10 min, 700 × *g*) of a second sucrose homogenate was centrifuged at 21,000 × *g* for 15 min. A second aliquot was spun (21,000 × *g*) for 60 min. Third and fourth aliquots were sonicated for 15 and 30 min, respectively, prior to centrifugation (21,000 × *g*, 60 min). The fifth aliquot was treated with N-butanol (20%, 1 hr, 0°C) according to the standard technique for butanol treatment outlined by Morton (1955). The butanol treated aliquot was also centrifuged for 1 hr at 21,000 × *g*, and the aqueous and butanol phases separated. The supernatants thus produced were all dialyzed against distilled water, diluted to a constant volume, and assayed for carbonic anhydrase activity.

Excavation rate and carbonic anhydrase content of clionid sponges

If clionid carbonic anhydrase is involved in the physiological mechanism of excavation, it is probable that the excavation rate is related to the concentration of the enzyme in the sponge. The difficulties involved in determining the excavation rate of clionid sponges were circumvented by developing an alternative technique (Hatch, 1974). In that as much as 90% or more of the substratum is excavated in the form of carbonate chips (Warburton, 1958), the weight of the chips expelled per unit time was used as an estimate of excavation rate.

Procedure. Alpha-, beta-, and gamma-form specimens of *Cliona celata* were cleaned of epibiota, positively identified as previously described, and placed in petri dishes in a flowing sea-water table. Each day expelled chips were collected on Whatman #4 filter paper, rinsed with distilled water and air dried for determination of calcium carbonate weight. The excavation rate was recorded as mg $CaCO_3$ expelled per day (averaged over 4 days), and the sponges were divided into actively excavating (25–50 mg $CaCO_3$/day), slowly excavating (1–25 mg $CaCO_3$/day), and non-excavating (no detectable carbonate chips). Four specimens from each group were extracted with butanol as previously described and assayed for carbonic anhydrase activity. In addition, gamma-form cortical and medullary tissues were extracted and assayed separately.

Effects of carbonic anhydrase inhibition on excavation rate

In order to establish a possible relationship between the physiological mechanism of penetration and carbonic anhydrase, the excavation rate of *Cliona celata* was determined in the presence of acetazolamide, a specific inhibitor of this enzyme.

Procedure. Thirty actively excavating alpha-form specimens of *Cliona celata* within *Mercenaria mercenaria* valves were freed of epibiota, identified, and returned to a flowing sea-water table as previously described. Through visual inspection of chip production over the course of a week, the 10 most active sponges were selected and arranged convex side up in petri dishes for chip collection (Hatch, 1974).

An inhibitor stock solution was prepared by dissolving sodium acetazolamide in Millipore-filtered sea water (1×10^{-4} M) and adjusting the pH back to that of the sea water system (8.26) with HCl. Inhibitor solution was introduced into flowing sea water through a metered-drip intravenous-infusion apparatus at a rate sufficient to maintain the desired acetazolamide concentration.

The excavation rate was determined over two 24 hr periods under the following conditions: 2.5 l/min sea water flow-through, 0.25 l/min flow-through with 2.25 l/min recirculation, and 0.25 l/min flow-through with 2.25 l/min recirculation in the presence of 10^{-5} and 10^{-6} M acetazolamide. The recirculation was necessitated by the sponges' requirement for a minimum current velocity and economic constraint on producing a 10^{-4} M inhibitor concentration with a large flow-through.

At the start of each determination sponges were carefully transferred under water into clean petri dishes. Expelled chips were collected after 24 hr as previously described.

Effect of carbonic anhydrase inhibition on in vitro metabolic rate

The possibility of toxic secondary effects of acetazolamide on the excavating ability of *Cliona celata* was investigated with *in vitro* respirometry utilizing a Gilson differential respirometer.

Procedure. The medullary tissue of beta-form specimens of *Cliona celata* was dissected from between the attached closed valves of *Mercenaria mercenaria*. The tissue was pooled, rinsed in running sea water, and teased into fragments less than 1 mm on a side.

Approximately 1 cm^3 of this tissue was placed in each Gilson reaction flask along with 3 ml of Millipore-filtered sea water. One half of one ml of a 20% w/v KOH solution was used as the CO_2 absorbant. One milliliter of the inhibitor solution (8.8×10^{-3} or 8.8×10^{-4} mg sodium acetazolamide/ml Millipore-filtered sea water, pH 8.26) was added to the side arm of each reaction flask. Fourteen replicates of each inhibitor concentration were run.

The flasks were equilibrated for 30 min, after which six readings were taken at 5-min intervals to establish the control respiratory rate. The inhibitor solution was then tipped into the sea water containing the sponge tissue and the O_2 consumption in the presence of 10^{-5} and 10^{-6} M acetazolamide was established with six additional readings at 5-min intervals. The total protein concentration of each flask was then determined by UV absorbance (Warburg and Christian, 1942).

Effects of carbonic anhydrase inhibition on papillary contraction

The ability of clonid sponges to respond to chemical sitimuli with papillary contraction has been documented (Emson, 1966). It is likely that papillary contraction and the resulting restriction of ostial and oscular openings could indirectly inhibit excavation by limiting the flow of sea water through the sponge. *In vivo* polarographic respirometry was utilized to reflect the degree of papillary contraction elicited by sodium acetazolamide.

Procedure. Alpha-form specimens of *Cliona celata* contained within *Mercenaria mercenaria* valves were selected to provide the maximum amount of respiring tissue that could be isolated by papillary contraction. Three individual valves were cleaned of epibiota and pooled for each experimental determination.

The oxygen consumption of the sponge was determined in a flow-through system in which sea water (1000 ± 2 ml/min) was passed first over the sponges in a sealed lucite chamber and then over a polarographic oxygen electrode coupled to a strip-chart recorder. The control respiratory rate was recorded for 30 min, after which an inhibitor stock solution (10^{-3} M sodium acetazolamide in sea water) was introduced into the inlet flow at 1 ml/min for an additional 30-min period. The inhibitor stock solution was then increased to 10^{-2} M and a final 30-min record of the respiratory rate was obtained.

Papillae were then induced to contract in response to a mechanical stimulus (tapping the lucite chamber), checking the function of the apparatus and the responsiveness of the sponges. Three replicates were run in this manner at 17.5° C. Oxygen consumption, indicative of the state of papillary contraction, was calculated from the difference in O_2 saturation of the sea water before and after it had passed over the sponges.

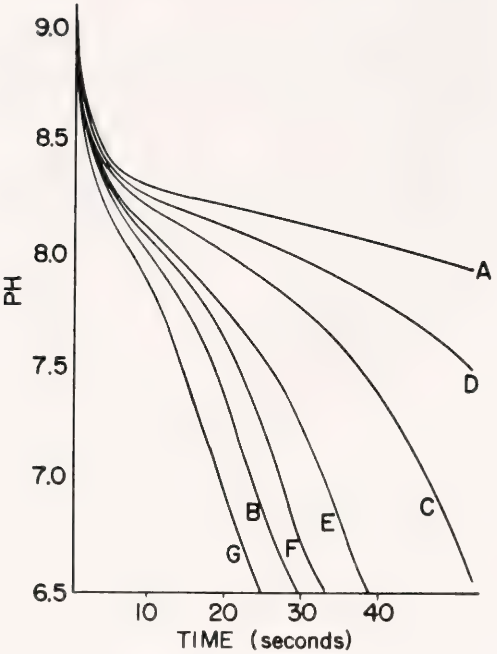


FIGURE 1. Clionid carbonic anhydrase reaction curves. Changes in pH are plotted against time for: A—distilled water control, B—sucrose extract prior to centrifugation, C—supernatant of sucrose extraction after centrifugation for 15 min at 21,000 × g, D—supernatant of sucrose extraction after centrifugation for 60 min at 21,000 × g, E—supernatant of sucrose extraction with 15 min of sonication prior to centrifugation (60 min, 21,000 × g), F—supernatant of sucrose extraction with 30 min of sonication prior to centrifugation (60 min, 21,000 × g), G—supernatant of sucrose extraction after treatment with N-butanol prior to centrifugation (60 min, 21,000 × g). Each line represents the mean of three runs.

RESULTS

Carbonic anhydrase activity was evident in crude water extracts. This activity was, however, largely removed by centrifugation, suggesting that the enzyme was not in cytoplasmic solution. The majority of enzyme activity was found in the precipitate from centrifugation at 21,000 × g. Spinning for 60 min removed more activity from the supernatant than spinning for 15 min. Sonication prior to centrifugation reduced the loss of activity from the supernatant. Finally, treatment with butanol precluded loss of enzyme activity from the supernatant during centrifugation even at 48,000 × g for several hours. The butanol phase contained no measurable carbonic anhydrase activity.

Excavation rate and carbonic anhydrase content

The enzymatic rate from the assay of each sponge is shown in Table I as enzyme units/mg of protein assayed. As can be seen from the data, there is a tendency for the more rapidly excavating alpha-form sponges to contain a higher concentration of carbonic anhydrase. The difference between the 25 and 50 mg/day group and the non-excavating group is of marginal significance (Student's t-test, $t = 1.16$, $0.10 < P < 0.15$). The difference between the carbonic anhydrase activities of the rapidly excavating sponges and gamma form medullary tissues was, however, highly significant (Student's t-test, $t = 9.01$, $P < 0.005$).

TABLE I

Carbonic Anhydrase content of alpha, beta, and gamma forms of Cliona celata.

	Sponge #	Milligrams protein/ml	E.U./mg*	Mean	s.e.
Alpha form 0 mg $CaCO_3$ excavated/day	1 2 3 4	1.93 1.87 1.96 1.79	3.1 26.1 29.9 26.0	21.28	3.55
Alpha form 1–25 mg $CaCO_3$ excavated/day	5 6 7 8	0.75 1.31 1.96 1.70	30.5 31.8 16.2 17.5	24.00	2.40
Alpha form 25–50 mg $CaCO_3$ excavated/day	9 10 11 12	1.83 1.93 1.82 2.00	22.9 30.4 23.3 25.1	25.49	1.00
Beta form 0 mg $CaCO_3$ excavated/day	13 14 15 16	1.85 1.83 1.83 1.89	11.0 28.3 3.8 36.9	20.00	4.41
Gamma form cortex 0 mg $CaCO_3$ excavated/day	17 18 19 20	1.79 1.80 1.91 1.61	37.6 36.8 39.1 41.8	38.83	0.70
Gamma form medulla 0 mg $CaCO_3$ excavated/day	21 22 23 24	1.89 1.87 2.01 1.57	16.6 13.4 11.3 15.2	14.13	1.42

* E.U. (enzyme unit) = 1 μM H^+/min.

Effects of carbonic anhydrase inhibition on excavation rate

The effects of enzyme inhibition on excavation rate are presented in Table II. There was considerable variability in the amount of carbonate excavated by the sponges during the two determinations. In spite of this variability, there was a profound effect on the excavation rate in the presence of carbonic anhydrase inhibition. In the presence of a 2.5 l/min flow-through, the average rate was 44.6 ± 4.8 mg of $CaCO_3$/day. When the flow-through was reduced to 0.25 l/min and the water was recirculated at 2.25 l/min, there was a slight, statistically insignificant decrease in the excavation rate to 41.2 ± 5.2 mg of $CaCO_3$. These results indicate that the reduced flow-through rates necessary to maintain the required inhibitor concentration may lower the excavation rate slightly, even when the current across the sponges is augmented by recirculating the sea water. In the presence of 10^{-6} M acetazolamide, however, there was a profound and statistically significant decrease in the excavation rate (13.6 ± 5.2 mg $CaCO_3$/day per sponge). In the presence of 10^{-5} M acetazolamide the excavation rate was further reduced to 3.0 ± 1.0 mg $CaCO_3$/day for each sponge. The total weight of the carbonate excavated by all of the sponges in each experiment demonstrates the same marked effect of the inhibitor on the excavation rate. The controls produced 823 mg $CaCO_3$/day while the same sponges in the presence of 10^{-6} and

TABLE II

Excavation rate of Cliona celata *in the presence of carbonic anhydrase inhibition. (Experimental condition: A = 2.50 l/min fresh sea water through-flow, B = 0.25 l/min fresh sea water through-flow augmented with recirculation (2.25 l/min) to an apparent flow of 2.50 l/min, C = as B with sodium acetazolamide added to a final concentration of 10^{-5} M, D = as B with 10^{-6} M acetazolamide.*

Experimental condition	Milligrams of calcium carbonate removed per day per sponge												
	Run one										$\bar{X}$	s.e.	Σ
A	41	60	4	61	34	39	71	72	53	59	49.4	6.5	494
B	54	18	36	99	46	18	90	84	11	66	43.2	9.4	432
C	00	00	02	01	03	04	05	05	06	07	03.3	0.78	033
D	16	17	12	19	10	08	16	12	11	13	13.4	1.1	134
	Run two												
A	55	44	34	24	33	00	73	63	17	56	39.9	7.1	399
B	40	20	23	31	37	37	43	73	32	53	39.1	4.9	389
C	00	01	01	02	00	07	05	04	07	00	0.27	0.89	027
D	18	19	15	19	08	05	05	21	14	14	13.8	1.7	138

Experimental condition	Total	Mean	Standard error	Number of specimens
A	893 mg	44.6 mg	4.8 mg	20
B	823 mg	41.2 mg	5.2 mg	20
C	060 mg	03.0 mg	1.0 mg	20
D	272 mg	13.6 mg	5.2 mg	20

10^{-5} M acetazolamide produced only 272 and 60 mg of calcium carbonate, respectively.

From these data it can be seen that the presence of acetazolamide does, in fact, inhibit the ability of *Cliona celata* to excavate calcium carbonate from the valves of *Mercenaria mercenaria*.

Effects of carbonic anhydrase inhibition on in vitro *metabolic rate*

The *in vitro* metabolic rate of the sponges remained unchanged by the addition of acetazolamide. The mean respiratory rate, in mm^3 O_2 consumed/100 mg of soluble protein, is shown graphed against time in Figure 2. From this graph it can be seen that the difference in metabolic rate in the presence and absence of acetazolamide falls within the standard error of the measurements. Thus, the effect of 10^{-5} and 10^{-6} M acetazolamide on the excavating ability of *Cliona celata* cannot be attributed to an inhibition of the sponge's metabolism.

Effects of carbonic anhydrase inhibition on papillary contraction

The *in vivo* metabolic rate was also unaffected by acetazolamide. Figure 3 shows oxygen consumption of the sponges (in μl O_2/min.) in the presence of acetazolamide. From this figure it can be seen that several minutes are required for the sponge to re-expand its papillae and resume normal oxygen consumption

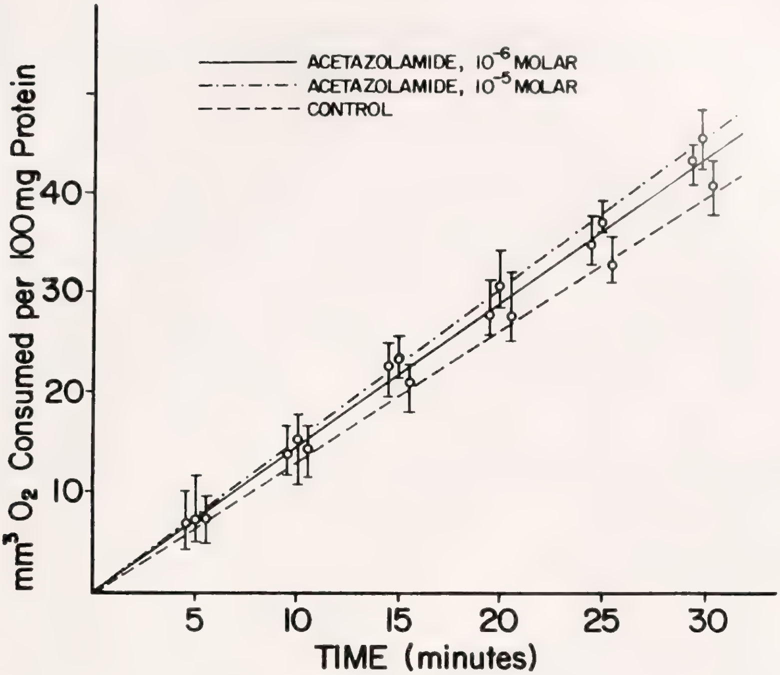


FIGURE 2. The effects of carbonic anhydrase inhibition on *in vitro* basal metabolism.

after handling. The small peaks just before T_0 may represent an increase in oxygen consumption resulting from an oxygen debt incurred during the handling period. No apparent difference in oxygen consumption can be seen from these graphs, indicating no detectable papillary contraction occurs in the presence of the concentrations of acetazolamide used to inhibit excavation. The mechanical tap, however, produces a dramatic change in oxygen consumption as the papillae contract in response to this stimulus.

Visual observation during the course of the experiment confirms that no papil-

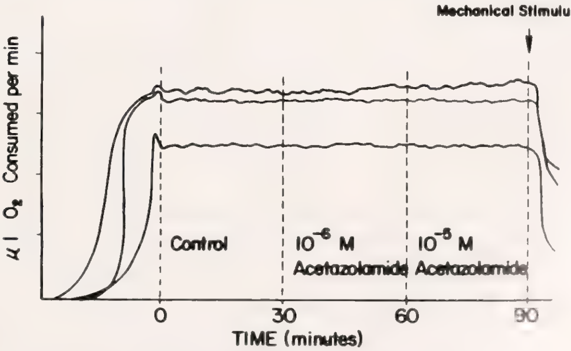


FIGURE 3. The effects of carbonic anhydrase inhibition on papillary contraction and *in vitro* respiratory rate. Respiratory rate of three pooled sponges is shown for three experimental runs. (Baseline rate established for 30 min prior to the addition of 10^{-6} and 10^{-5} M acetazolamide.) The mechanical stimulus served as an equipment check (note rapid drop in respiratory rate with papillary contraction).

lary contraction occurs in response to acetazolamide. It can then be concluded that the inhibition of clionid excavation by acetazolamide is not mediated by the ability of the sponge to detect and respond to inhibition by papillary contraction.

DISCUSSION

The exact manner by which carbonic anhydrase might mediate the finely controlled dissolution of the substratum by clionid etching cells is unclear. Unlike the carbonic anhydrase of *Urosalpinx cinerea,* which is in cytoplasmic solution in the microvillar zone of the accessory boring organ (Smarsh *et al.,* 1969) or released in the ABO secretion (Carriker and Chauncey, 1974), clionid carbonic anhydrase appears to be associated with mitochondrial-sized particles.

The presence of carbonic anhydrase in the tissues of *Cliona celata,* however, indicates that the sponge has the capacity to greatly increase the speed of the reversible reaction $H_2O + CO_2 \rightleftharpoons H^+ + HCO_3^-$ and accelerate the precipitation or dissolution of $CaCO_3$ by shifting the solubility equilibria. Both the tendency for rapidly excavating sponges to have higher carbonic anhydrase activities than non-excavating sponges and the inhibition of excavation rate by specific enzyme inhibitors strongly suggest the participation of this enzyme in the excavation process.

The enzyme concentration of gamma-form sponges presents a problem. Although no substratum was present, the carbonic anhydrase concentration in the cortical tissues was much higher than in alpha- or beta-form sponges. The fact that the cortical tissues contained much higher concentrations of the enzyme than the medullary tissues suggests the enzyme may be localized in surface-lining cells. It is these cells that would come in contact with fresh substratum. The lower enzyme concentrations of beta-form sponges may also be attributed to a relative increase in non-excavating tissue.

Rutzler and Reiger (1973) demonstrated a prominent nucleolus, abundant ribosomes, and an extremely active golgi complex within the etching cells of clionid sponges, suggesting that these cells are involved in protein and carbohydrate synthesis even in advanced stages of plasmolysis. In addition, the filopodial basket contains numerous vesicles as well as a flocculent secretory product. Although they never saw these vesicles emptying their contents to the outside (Rutzler, personal communication), they speculated that the flocculent material observed by them within the etching cells, and in the space between the cell and substratum, is responsible for the penetrating activity of these cells.

It is unlikely, however, that the secretory product seen by Rutzler and Reiger represents carbonic anhydrase, as a direct catalytic breakdown of the substratum by the enzyme has been ruled out (Carriker and Chauncey, 1974). Clionid carbonic anhydrase activity can be removed from crude aqueous extracts by centrifugation, suggesting the enzyme might be associated with some subcellular structure (Morton, 1955). The enzyme could occur, therefore, in solution within a limiting semipermeable membrane (Schneider, 1953) or intimately associated with the insoluble lipoprotein of the membrane (Morton, 1953, 1954). The fact that sonic or butanol disruption of membranes precludes loss of enzyme activity during centrifugation supports the hypothesis that the enzyme is contained within, or is associated with, the membrane of vesicles within the filopodial basket.

There are several equally plausible mechanisms for the participation of carbonic anhydrase in the penetration of both organic and inorganic components of shell. First, carbonic anhydrase, within the filopodial basket, could accomplish the dissolution of calcium carbonate by simply providing hydrogen ions for transport across the membrane. Thus, the mechanism may be similar to that found in parietal cells (Maren, 1967) in which the H^+ ions are transported across the membrane along with Cl^- ions.

It is equally valid to speculate that the mechanism of penetration involves carbonic anhydrase in a role similar to that found in mammalian kidney (Maren, 1967). In clionid etching cells the exchange of hydrogen for bicarbonate ions would result in both the dissolution of the substratum and a lowering of the pH, with possible optimization for the enzymes responsible for the dissolution of the organic matrix.

In addition, carbonic anhydrase has been implicated in the transmembrane transport of Ca^{2+} ions (Istin and Kirschner, 1968; Ehrenspeck *et al.*, 1971). Thus, the penetration mechanism may involve a transmembrane flux of Ca^{2+} ions as well as a simple pH shift.

It is also possible that the H^+ ions resulting from the activity of carbonic anhydrase participate only indirectly in the dissolution of the substratum by providing pH optimization for the activity of chelating agents and/or the enzyme responsible for the breakdown of the organic matrix. The experimental determination of a chelating agent within the etching cell or its penetrative agent must be accomplished to confirm this hypothesis.

In summary, carbonic anhydrase in *Cliona celata*, coupled with the demonstration that the concentration of the enzyme in the sponge tissues is related to the excavating activity of the sponge, suggests this enzyme is involved in the physiological mechanism of penetration. This conclusion is supported by evidence that enzyme inhibition results in inhibition of the excavating ability of the sponge. The inability of the sponge to detect and respond to acetazolamide with papillary contraction or a depressed metabolic rate indicates that this inhibition of excavation rate is neither mediated by a behavioral response nor by a generalized metabolic inhibition.

This work constituted part of a doctoral dissertation submitted to the Department of Biology, Boston University, and was conducted in conjunction with the Boston University Marine Program at the Marine Biological Laboratory, Woods Hole, Mass. It was supported in part by a graduate fellowship and by aid from grants through the Boston University Marine Program.

SUMMARY

1. The marine burrowing sponge *Cliona celata* contains measurable carbonic anhydrase activity when assayed with a modified electrometric method.

2. When clionid tissues are extracted and centrifuged in isotonic sucrose, the majority of the enzyme activity is found in the mitochondrial size fraction, indicating the enzyme is membrane- or particle-bound.

3. Much of the enzyme activity can be released into solution with sonication. Treatment with isobutanol, which dissociates lipoprotein complexes, releases all of the enzyme activity into solution.

4. From a range of buffer solutions, 0.025 M trisaminomethane (pH 8.4–8.7) was chosen as the optimal buffer for the assay of clionid carbonic anhydrase.

5. There is a positive correlation between the excavating activity of the sponge (mg of $CaCO_3$ removed per day) and the level of carbonic anhydrase in sponge tissues. In addition, enzyme activity is concentrated in the cortical tissues of gamma-form *C. celata*. It is these tissues that are most likely to come in contact with fresh substrata in the form of shell fragments.

6. Sodium acetazolamide is capable of inhibiting the activity of clionid carbonic anhydrase *in vitro* with no apparent inhibition of the overall metabolism of the overall metabolism of the sponge.

7. Acetazolamide-induced inhibition of the sponge's carbonic anhydrase activity markedly reduces the ability of alpha-form *C. celata* to excavate calcium carbonate chips from the valves of *Mercenaria mercenaria.*

8. Similar concentrations of acetazolamide provoke no papillary contraction or reduction in oxygen consumption *in vivo*, indicating the reduction in excavation rate is not due to the ability of the sponge to detect the acetazolamide and shut down the flow of sea water through its choanosome. It appears, therefore, that the primary mechanism for the dissolution of calcium carbonate by *C. celata* involves a shift in the carbonate solubility product in the microenvironment of the etching cell, mediated through the activity of the enzyme carbonic anhydrase.

LITERATURE CITED

Addink, A. D. F., 1971. Carbonic anhydrase of *Sepia officinalis L. Chem. Physiol.*, **38**: 707–721.

Carriker, M. R., and H. H. Chauncey, 1974. Effect of carbonic anhydrase inhibition on shell penetration by the muricid gastropod *Urosalpinx cinerea*. *Malacologia*, **12**: 247–263.

Carter, M. J., 1972. Carbonic anhydrase: isozymes, properties, distribution, and functional significance. *Biol. Rev.*, **47**: 465–513.

Carter, M. J., D. J. Harvard, and D. S. Parsons, 1969. Electrometric assay of rate of hydration of CO_2. *J. Physiol., Lond.*, **204**: 60–62.

Chétail, M., D. Binot, and M. Bensalem, 1968. Organe de performation de *Purpura lapillus L.* (Murcidé): histochimie et histoenzymologie. *Cah. Biol. Mar.*, **9**: 13–22.

Chétail, M., and J. Fournié, 1969. Shell-boring mechanism of the gastropod, *Purpura lapillus L.*: a physiological demonstration of the role of carbonic anhydrase in the dissolution of $CaCO_3$. *Am. Zool.*, **9**: 983–990.

Cobb, W. R., 1969. Penetration of calcium carbonate substrata by the boring sponge *Cliona. Am. Zool.*, **9**: 783–790.

Cobb, W. R., 1971. Penetration of calcium carbonate by *Cliona celata*, a marine burrowing sponge. *Ph.D. thesis, University of Rhode Island*, 163 pages. *Diss. Abstr.* #72–9790.

Davis, R. P., 1963. The Measurement of Carbonic Anhydrase Activity. Pages 307–328 in D. Glick, Ed., *Methods of Biochemical Analysis.* (Vol. XI). Interscience, New York.

Ehrenspeck, G., H. Schraer, and R. Schraer, 1971. Calcium transfer across isolated avian shell gland. *Am. J. Physiol.*, **220**: 967–972.

Emson, R. H., 1966. The reaction of the sponge *Cliona celata* to applied stimuli. *Comp. Biochem. Physiol.*, **18**: 825–827.

Goreau, T. F., and W. D. Hartman, 1963. Boring sponges as controlling factors in the formation and maintenance of coral reefs. Pages 25–54 in R. F. Sognnaes, Ed., *Mechanisms of Hard Tissue Destruction.* Publ. No. 75, American Association for the Advancement of Science, Washington, D.C.

Gray, J. E., 1867. Notes on the arrangement of sponges with the description of new genera. *Proc. Zool. Soc. London*, 1867: 492–558.

Hancock, A., 1849. On the excavating powers of certain sponges belonging to the genus *Cliona:* with descriptions of several new species and an allied generic form. *Ann. Mag. Nat. Hist.*, (Ser. 2) **3**: 321–348.

Hatch, W. I., 1974. The implication of carbonic anhydrase in the physiological mechanism of penetration of carbonate substrata by the marine burrowing sponge *Cliona celata. Ph.D. thesis, Boston University*, 159 pages. *Diss. Abstr.* #75–12,251.

HEATFIELD, B. M., 1970. Calcification in echinoderms: Effects of temperature and Diamox on incorporation of calcium-45 *in vitro* by regenerating spines of *Strongylocentrotus purpuratus*. *Biol. Bull.*, **139**: 151–163.

HYMAN, L. H., 1940. Metazoa of cellular grade of construction—Phylum Porifera, The Sponges. Pages 284–364 in *The Invertebrates: Protozoa through Ctenophora* Vol. I. McGraw-Hill, New York.

ISTIN, M., AND J. P. GIRARD, 1970. Carbonic anhydrase and mobilization of calcium reserves in the mantle of lamellibranchs. *Calif. Tissue Res.*, **5**: 247–260.

ISTIN, M., AND L. B. KIRSCHNER, 1968. On the origin of bioelectric potentials generated by the fresh water clam mantle. *J. Gen. Physiol.*, **51**: 478–485.

LEIDY, J., 1889. The boring sponge *Cliona*. *Proc. Acad. Nat. Sci. Philadelphia,* **41**: 70–75.

MAREN, T. H., 1967. Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiol. Rev.*, **47**: 598–781.

MORTON, R. K., 1953. Alkaline phosphatase of milk: 2. Purification of the enzyme. *Biochem. J.*, **55**: 795–800.

MORTON, R. K., 1954. Purification of alkaline phosphatase of animal tissues. *Biochem. J.*, **57**: 595–603.

MORTON, R. K., 1955. Methods of Extraction of Enzyme from Animal Tissue. Pages 25–30 in S. P. Colowick and N. O. Kaplan, Eds., *Methods in Enzymology, Vol. I: Preparation and Assay of Enzymes.* Academic Press, New York.

NASSONOW, N., 1883. Zur Biologie and Anatomie der *Clione*. *Z. Wiss. Zool.,* **39**: 295–308.

OLD, M. C., 1941. Taxonomy and distribution of the boring sponges Clionidae along the Atlantic Coast of North America. *Chesapeake Biol. Lab. Publ. No. 44:* 1–30.

OSLER, E., 1826. On burrowing and boring marine animals. *Phil. Trans. R. Soc. Lond.*, **116**: 342–371.

ROSENBERG, A., A. JEAN, M. CHÉTAIL, AND J. FOURNIÉ, 1968. Intervention de l'anhydrase carbonique dans le méchanisme de performation des valves de Lamellibranches par *Purpura (Thais) lapillus L.* (Gastéropode, Prosobranche Murcidé). *C. R. Hebd. Seances Acad. Sci. Natur. Paris*, **266**: 944–947.

RUTZLER, K., AND G. REIGER, 1973. Sponge burrowing: fine structure of *Cliona lampa* penetrating calcareous substrata, *Mar. Biol.*, **21**: 144–162.

SCHNEIDER, W. C., 1953. Biochemical constitution of mammalian mitochondria. *J. Histochem. Cytochem.*, **1**: 212–233.

SMARSH, A., H. H. CHAUNCEY, M. R. CARRIKER, AND P. PERSON, 1969. Carbonic anhydrase in the accessory boring organ in the gastropod *Urosalpinx*. *Am. Zool.*, **9**: 967–982.

TOPSENT, E., 1888. Contribution á l'étude des *Clionides*. *Archs. Zool. Exp. Gen.* (Ser. 2) 1887–1890., 5 Supplémentaire: 5–165.

TURQUIER, Y., 1968. Recherches sur la biologie des cirripèdes acrothoraciques I. L'anhydrase carbonique et le méchanisme de perforation du substrat par *Trypetesa nassarioides* Turq. *Arch. Zool. Exp. Gen.*, **109**: 113–122.

VOSMAER, G. C. J., 1933–1935. The sponges of the Bay of Naples: Porifera Incalcarea. Pages 1–456 in E. D. Van Oort, Ed., *Capita Zoologica* (Vol. I). Martinus Nijhoff, the Hague, Netherlands.

WARBURTON, R., 1958. The manner in which the sponge *Cliona* bores in calcareous objects. *Can. J. Zool.*, **36**: 555–562.

WARBURG, O., AND W. CHRISTIAN, 1942. Isolierung and Kristallisation des Garungsferments Enolase. *Biochem. Z.*, **310**: 384–421.

WILBUR, K. M., AND N. G. ANDERSON, 1948. Electrometric and colorimetric determination of carbonic anhydrase inhibitors. *J. Biol. Chem.*, **176**: 147–154.

Reference: *Biol. Bull.*, **159**: 148–161. (August, 1980)

THE EFFECTS OF TEMPERATURE AND SALINITY ON THE OSMOTIC COMPOSITION OF THE SOUTHERN OYSTER DRILL, *THAIS HAEMASTOMA*

JANE E. HILDRETH[1] AND WILLIAM B. STICKLE

Department of Zoology and Physiology, Louisiana State University,
Baton Rouge, Louisiana, 70803

Temperature and salinity are two of the most important physical factors affecting estuarine organisms. In most studies on the effects of physical variables on estuarine organisms, all factors but one have been rigorously controlled and kept constant. Kinne (1971) argues that multifactorial experiments measuring the effects of two or more environmental factors acting in concert are necessary to understand the functioning of estuarine organisms under natural conditions.

Most previous work on the effects of temperature and salinity on osmoregulation of marine and estuarine invertebrates has been done using osmoregulating crustaceans (Verwey, 1957; Lockwood, 1960; Dehnel, 1962; Todd, 1963; Dehnel and Carefoot, 1965; Taylor *et al.*, 1977). These workers and others have found that most euryhaline invertebrates studied exhibit greater osmoregulatory capabilities in the lower portions of their normal temperature ranges (see Kinne, 1970, 1971, for review).

Many workers have investigated the effects of salinity on the blood solute concentrations of various molluscs, but the possible interaction of salinity and temperature on osmotic and ionic regulation in molluscs has not been studied thoroughly. The present study had two objectives: 1) to investigate the interaction of temperature and salinity on the osmotic composition of an osmoconforming marine gastropod *Thais haemastoma* and 2) to investigate the effects of long-term salinity fluctuations on this snail. Hemolymph osmolality and concentrations of Na^+, K^+, Mg^{2+}, and Cl^- under constant and fluctuating salinity were measured at 10°, 20°, and 30°C. In addition, percent tissue water and the level of ninhydrin positive substances (NPS) in the foot tissue were determined.

MATERIALS AND METHODS

Specimens of *Thais haemastoma* were collected during September and October, 1977, from Barataria Bay in the vicinity of Grande Isle, La., U.S.A. Ambient salinity at the time of collection varied from 24 to 28‰, water temperature between 26° and 30°C. Snails were returned to the laboratory and placed in aquaria containing Instant Ocean artificial seawater (Eastlake, Ohio) at room temperature and the same salinity as the collection site. Snails were brought to the initial salinity of each experiment by adjusting the salinity (S) 2‰ per day until the desired salinity was reached. The aquaria were then placed in a water bath (20° and 30°C experiments) or a cold room (10°C experiment) and held at this temperature-salinity combination for 14 days before simulated tidal cycles were begun. The oyster drills were held under constant illumination during the acclimation period and all experiments. Live oysters were available as food for the drills at all times.

---

[1] Present Address: Department of Zoology, Oregon State University, Corvallis, Oregon 97331.

Constant salinity

Snails were acclimated to 7.5, 10, 20, and 30‰ S for 5 weeks at 10° and 20°C and 4 weeks at 30°C. At the time of sampling snails were removed from acclimation aquaria and an ambient seawater sample taken. The shell was cracked near the heart and 50–300 μl of hemolymph collected in microcapillary tubes and placed in 400 μl plastic snap-cap centrifuge tubes. Hemolymph samples were centrifuged at 9000 × *g* for 5 min to pellet any particulate matter. The osmolality of hemolymph and seawater was determined with a WESCOR Vapor Pressure Osmometer. Hemolymph and water samples were diluted with deionized water for ion analyses. The foot was removed, blotted, and the weight determined; it was frozen on dry ice and lyophilized to constant weight for the determination of percent tissue water.

Chloride ion concentrations were determined with an Aminco Chloridometer, Na^+ and K^+ with a Coleman Flame Photometer, and Mg^{2+} with a Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer.

Ninhydrin positive substances (NPS) were determined in the foot tissue. The freeze dried foot was ground to a powder in a Wiley grinding mill and 10 mg tissue incubated 48 hr in 10 mls of 10% 5-sulfosalicylic acid to precipitate proteins and allow complete leaching of the NPS from the tissue. The samples were centrifuged at 12,100 × *g* for 15 min and NPS determined on a 0.1 ml aliquot of the supernatant by the method of Rosen (1957). Leucine was used as the standard. NPS levels are reported as μM NPS/g dry weight and as μM NPS/g tissue water.

Salinity fluctuation experiments

Salinity fluctuations simulated diurnal tidal cycles of 24 hr. A dilution apparatus described by Stickle and Ahokas (1974) was used to simulate tidal cycles (salinity regimes) of 30–10–30‰ and 10–30–10‰ S. The salinity was raised or lowered during the first 10 hr of the cycle, held at this salinity for a 2-hr slack-water period, then returned to the initial salinity during the next 10 hr. After a second 2-hr slack water period the cycle was repeated daily for a total of 21 days. Both cycles were run at 10°, 20°, and 30°C.

Snails were killed and hemolymph samples taken on Days 1, 3, 6, 9, 12, 15, 18, and 21 in the 10°C experiment. Samples were taken on the same days in the other two experiments with the following exceptions: 1) only snails under the 30–10–30‰ salinity regime were sampled on Days 6 and 15 of the 30°C experiment and 2) no samples were taken on Day 9 of the 20°C experiment. These sample periods were omitted to reduce the numbers of animals used in the experiments.

Statistical analysis: Constant salinity

Because the salinity of aquaria varied by as much as 2‰ among the three temperatures tested, data were standardized by expressing osmolality and ion concentrations as the hemolymph concentration minus the seawater concentration. In this way observed differences are due to temperature effects, not to minor variations in salinity among the three experiments.

An analysis of variance (ANOVA) was run to test the effects of temperature, salinity, and temperature-salinity interaction (a 3 × 4 factorial design) on present tissue water, hemolymph osmolality, Cl^-, Na^+, Mg^{2+}, and K^+ concentrations, μM NPS/g dry weight, and μM NPS/g tissue water of acclimated snails. Duncan's

TABLE I

Mean hemolymph (HL)-minus-seawater osmolality and ion concentrations of Thais haemastoma *held at 12 temperature-salinity combinations (mean percent tissue water in the foot is also given for each temperature-salinity combination). Values given are mean ± standard error (n). Columns or rows joined by the same bar are not significantly different. An * indicates hemolymph-minus-seawater osmolality is significantly different from 0 at the 5% level.*

	7.5‰	10‰	20‰	30‰
Osmolality (mOsm)				
Water	219	292	584	876
HL 10°C	26 ± 17 (4)	38 ± 13 (5)*	135 ± 16 (5)*	23 ± 1 (4)*
20°C	5 ± 5 (5)	27 ± 2 (5)*	9 ± 1 (5)*	1 ± 2 (5)
30°C	8 ± 5 (5)*	4 ± 2 (4)*	29 ± 10 (5)	−34 ± 12 (4)
Tissue Water (%)				
Water				
HL 10°C	78 ± 1 (6)	78 ± 2 (5)	73 ± 2 (4)	74 ± 1 (2)
20°C	77 ± 1 (5)	76 ± 0 (5)	76 ± 0 (4)	76 ± 1 (4)
30°C	77 ± 0 (5)	77 ± 1 (4)	75 ± 1 (5)	72 ± 1 (4)
Cl^- (mM)				
Water	117	156	312	469
HL 10°C	−6 ± 17 (4)	−20 ± 13 (4)	74 ± 22 (5)	−17 ± 8 (2)
20°C	−24 ± 4 (5)	−42 ± 4 (4)*	−22 ± 7 (5)	8 ± 11 (5)
30°C	−20 ± 3 (5)	−15 ± 3 (4)	3 ± 15 (5)	−90 ± 2 (3)*
Na^+ (mM)				
Water	98	130	261	391
HL 10°C	52 ± 15 (5)	29 ± 28 (4)	20 ± 5 (5)	−7 ± 51 (2)
20°C	−14 ± 9 (5)	−14 ± 2 (4)*	40 ± 3 (5)	−37 ± 11 (5)
30°C	10 ± 16 (5)	4 ± 6 (4)	2 ± 7 (5)	4 ± 11 (3)
Mg^{2+} (mM)				
Water	11.4	15.2	30.4	46.6
HL 10°C	14.9 ± 2.0 (5)*	10.9 ± 3.0 (4)*	−11.5 ± 1.6 (5)*	−1.0 ± 2.0 (2)
20°C	10.4 ± 1.0 (5)*	8.7 ± 0.8 (4)*	8.6 ± 0.8 (5)*	2.7 ± 2.9 (5)
30°C	1.0 ± 2.0 (5)	3.2 ± 0.7 (4)	0.2 ± 0.7 (5)	−2.4 ± 2.5 (3)
K^+ (mM)				
Water	2.2	2.8	5.6	8.5
HL 10°C	3.5 ± 0.7 (5)	3.7 ± 1.0 (3)	1.8 ± 0.2 (4)*	3.0 ± 0.0 (2)
20°C	4.3 ± 0.7 (5)	3.5 ± 0.8 (4)*	1.9 ± 0.1 (5)*	1.2 ± 0.6 (5)
30°C	2.2 ± 0.8 (5)	1.5 ± 0.2 (4)*	4.1 ± 2.0 (5)	9.4 ± 2.0 (3)

New Multiple Range Test was used to find significance of differences among temperature means. A preliminary analysis of variance showed no sex effect in either the constant or fluctuating salinity conditions, so sex was not included in any further ANOVA's. Snail hemolymph osmolality and ion concentrations were compared with seawater values by *t*-tests for each temperature-salinity combination. A significant difference ($P \leq 0.05$) would indicate regulation of hemolymph osmolality or ion concentrations as compared to seawater values.

Statistical analysis: Salinity fluctuation experiments

Data from the salinity fluctuation experiments were also standardized by expressing hemolymph osmolality and ion concentrations as hemolymph minus seawater concentrations.

An ANOVA was run testing the effects of temperature, salinity regime, high versus low stage of fluctuation cycle, and time on percent tissue water, hemolymph osmolality, Cl^-, Na^+, Mg^{2+}, and K^+ concentrations of snails under fluctuating salinity regimes. For the ANOVAs time was broken into four classes: Days 1 and 3, 6 and 9, 12 and 15, and 18 and 21. This is a $3 \times 2 \times 2 \times 4$ factorial design. Duncan's New Multiple Range Test was used to detect significant differences ($P \leq 0.05$) among means of the dependent variables over time and to compare the three temperature experiments. Maximum R-square regressions of all dependent variables against time were performed for each temperature-salinity-regime stage-of-cycle combination. Regression lines are plotted as predicted values with standard errors. All statistical analyses were done using the Statistical Analysis System, Version 76.6 (Barr *et al.*, 1976).

RESULTS

Constant salinity

Temperature, salinity, and temperature-salinity interaction all had significant effects ($P \leq 0.05$) on the difference between hemolymph and seawater osmolality. Mean values for snails held at 7.5, 10, 20, and 30‰ salinity and 10°, 20°, and 30°C are given in Table I. Hemolymph osmolality increased as salinity increased. The *t*-test comparisons of hemolymph osmolality against seawater osmolality indicate a significant difference between hemolymph and seawater osmolality at 10, 20, and 30‰ and 10°C (Table I). There are statistically significant differences at two salinities under both the 20° and 30°C regimes, but the differences are smaller. Hemolymph osmolality was maintained slightly above seawater values at 10°C and was isosmotic or slightly hyperionic at 20° and 30°C. Percent tissue water was unaffected by temperature and decreased as ambient salinity and hemolymph osmolality increased (Table I).

Hemolymph concentrations of all ions studied increased as salinity increased under steady state conditions. Temperature, salinity, and temperature-salinity interactions all had significant effect ($P \leq 0.05$) on the difference between hemolymph and seawater Cl^-, Na^+, and Mg^{2+} concentrations (Table I). Hemolymph Cl^- concentrations were generally lower than seawater values. At 10°C hemolymph Na^+ concentrations were greater than seawater concentrations, while hemolymph Na^+ concentrations did not differ from ambient levels at 20° and 30°C. Hemolymph $[Mg^{2+}]$ was isionic or slightly hyperionic to ambient seawater at most temperature-salinity combinations examined. The *t*-test comparisons indi-

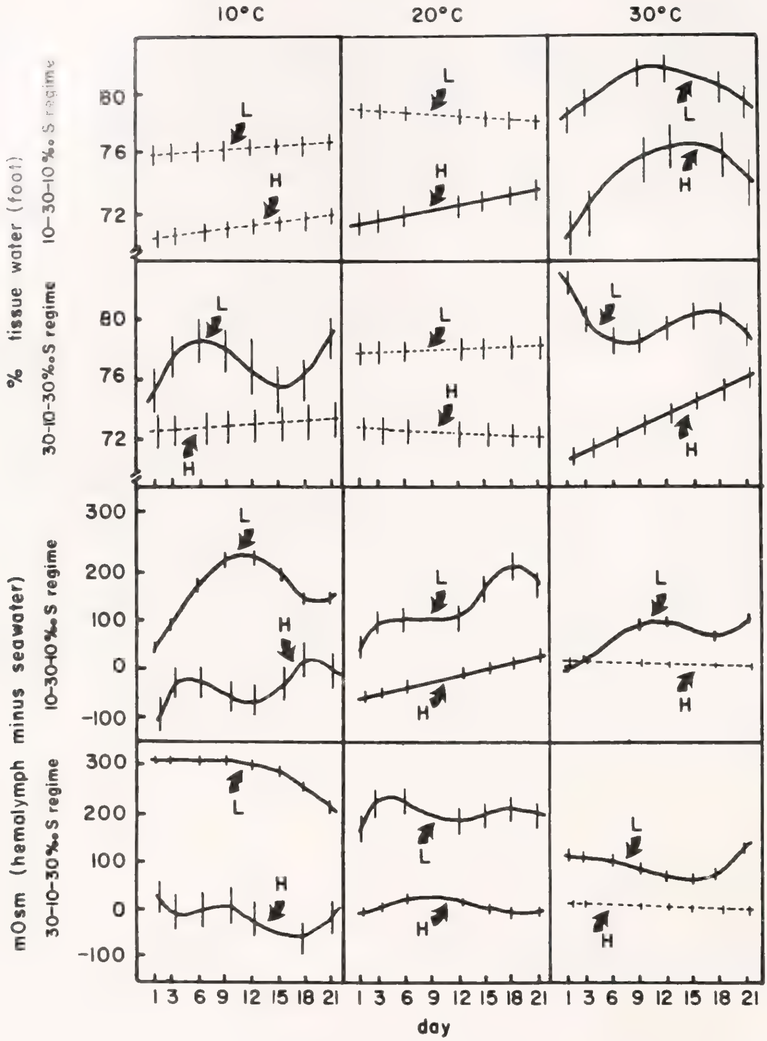


FIGURE 1. Regression lines for hemolymph osmolality of *Thais haemastoma* expressed as hemolymph osmolality minus seawater osmolality at the high (H) and low (L) phases of salinity cycles. Percent body water of the foot at high and low phases of salinity cycles are also given. Lines are presented for 10–30–10‰ and 30–10–30‰ S cycles at 10°, 20°, and 30°C. Vertical lines represent standard error at each sample point.

cated significant differences between hemolymph and seawater Mg^{2+} concentrations at 7.5, 10, and 20‰ S at both 10° and 20°C. Although neither temperature nor salinity had significant effects on K^+ hemolymph-minus-seawater differences, the effect of temperature-salinity interactions was significant ($P \leq 0.05$). Hemolymph $[K^+]$ increased with increasing seawater concentration and was unaffected by temperature. The *t*-test comparisons for difference between seawater and hemolymph $[K^+]$ showed significant differences at 10 and 20‰ salinity.

Fluctuating salinity experiments

Hemolymph osmolality tracked that of ambient seawater at all three temperatures under both salinity regimes. Hemolymph tended to be isosmotic or slightly hyposmotic during high salinity slackwater periods and to be hyperionic during low salinity slackwaters. This trend was most evident at 10°C. Figure 1 shows maximum R-square regression lines of hemolymph-minus-seawater osmolality for high and low salinity sample periods at 10°, 20°, and 30°C under both salinity regimes. Broken lines indicate nonsignificant, best-fit lines.

Analysis of variance (ANOVA) showed temperature, salinity regime, stage of cycle (high or low), time, and all two way interactions of these variables to have significant ($P \leq 0.05$) effects on the difference of osmolality between hemolymph and seawater. Duncan's New Multple Range Test showed that hemolymph-minus-seawater osmolality was greatest at 10°C, intermediate at 20°C, and least at 30°C over the 21-day salinity fluctuation experiment. Duncan's test also showed that hemolymph-minus-seawater osmolality was greater in snails under the 30–10–30‰ S fluctuating salinity regime than in snails under the 10–30–10‰ S regime. A large positive difference at low salinity slackwaters, as was observed at 10°C and to a lesser extent at 20° and 30°C (Fig. 1), indicates snail hemolymph was not closely tracking ambient levels during this phase of the salinity cycle.

Tissue water in the foot varied inversely with seawater and hemolymph osmolality, increasing with decreasing salinity (Fig. 1). ANOVA showed that temperature, stage of cycle, and time had significant effects ($P \leq 0.05$) on tissue water content. Tissue water was greatest at 30°C, intermediate at 20°C, and least at 10°C according to Duncan's test. This difference in tissue water content was obvious at the time of sampling. Bleeding of the snails was more difficult at 10°C than at the other temperatures and at 30°C the foot hemolymph was evident even after blotting the severed foot.

Temperature and stage of cycle, but not salinity regime, had significant ($P \leq 0.05$) effects of hemolymph Cl^- values expressed as hemolymph-minus-seawater concentrations when salinity was fluctuated. All two way interactions, including those involving salinity regime, were significant. Duncan's New Multiple Range Test showed hemolymph-minus-seawater values to be greatest at 10°C and least at 30°C. Hemolymph-minus-seawater $[Cl^-]$ was greater than zero, indicating hemolymph Cl^- was maintained above seawater values at most low-salinity sample points at 10° and 20°C (Fig. 2). At 30°C hemolymph-minus-seawater Cl^- concentration was close to zero at both high and low salinity sample points, *i.e.*, hemolymph Cl^- tracked ambient water concentrations closely at 30°C.

Somewhat similar trends were observed for hemolymph-minus-seawater Na^+ concentrations. Figure 2 shows that at 10°C hemolymph-minus-seawater Na^+ concentration was consistently greater than zero during the low-salinity sample periods while there was little difference between hemolymph and seawater concentrations at high-salinity sample points. The data were more variable at the higher temperatures but tend to show hemolymph Na^+ concentration tracking ambient levels more than at 10°C. ANOVA showed that temperature, salinity regime, stage of cycle, time, and all two way interactions involving these variables, except stage of cycle by time interaction, have significant effects on hemolymph-minus-seawater $[Na^+]$. Duncan's test showed hemolymph-minus-seawater Na^+ to be greatest at 10°C and least at 30°C and to be greater in snails under the 30–10–30‰ salinity regime (30‰ S acclimation) than in those under the 10–30–10‰ S regime.

Hemolymph Mg^{2+} concentrations showed trends similar to those observed for the other ions studied. Hemolymph $[Mg^{2+}]$ was hyperionic during low-salinity sample periods and usually isionic or slightly hypoionic during high-salinity sample periods (Fig. 3). Temperature, stage of cycle, time, and all two way interactions

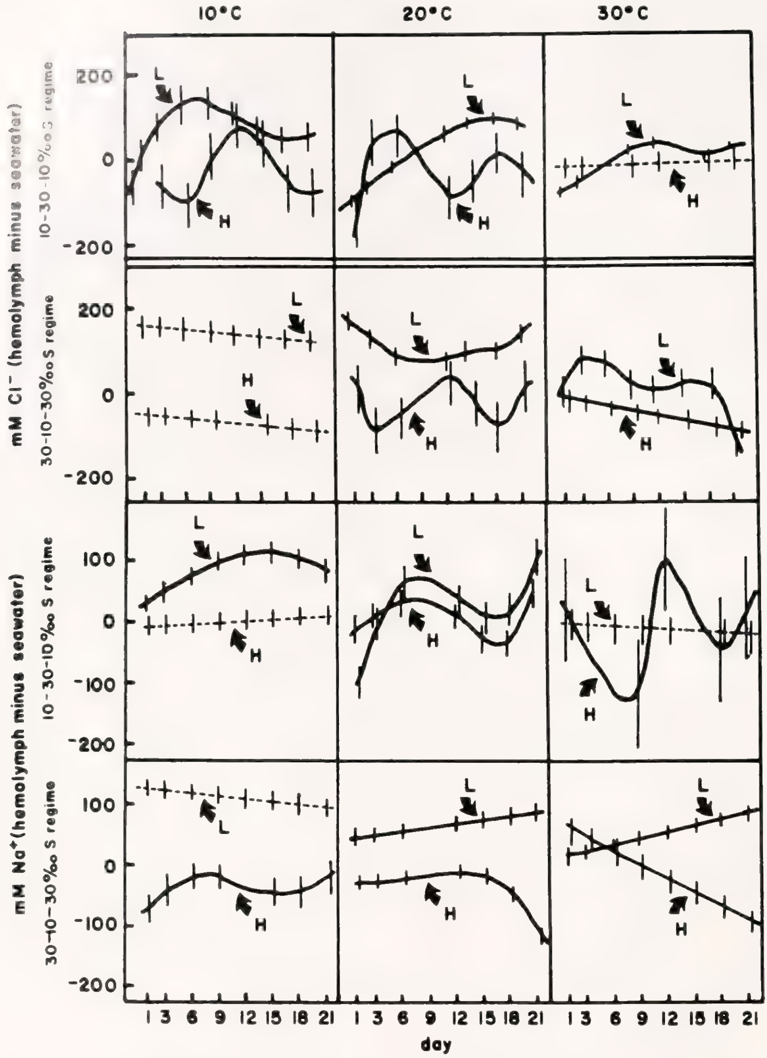


FIGURE 2. *Thais haemastoma.* Regression lines for hemolymph Cl^- and Na^+ concentration of *Thais haemastoma* expressed as hemolymph-minus-seawater concentration at the high (H) and low (L) phases of salinity cycles. Lines are presented for 10–30–10‰ and 30–10–30‰ S cycles at 10°, 20°, and 30°C. Vertical lines represent standard error at each sample point.

tested except stage of cycle by time had significant effects ($P \leq 0.05$) on hemolymph-minus-seawater Mg^{2+}. Duncan's test showed that hemolymph-minus-seawater [Mg^{2+}] was greater at 10°C than at 20° and 30°C.

Hemolymph [K^+] also tended to be isionic to ambient seawater during high-salinity sample periods and to be hyperionic at low-salinity sample points (Fig. 3). Hemolymph K^+ was less hyperionic at low-salinity sample points at 30°C than at the other temperatures, indicating it tracked ambient levels to a greater extent. Temperature, salinity regime, and stage of cycle all significantly affected ($P \leq 0.05$) hemolymph K^+ expressed as hemolymph-minus-seawater concentration,

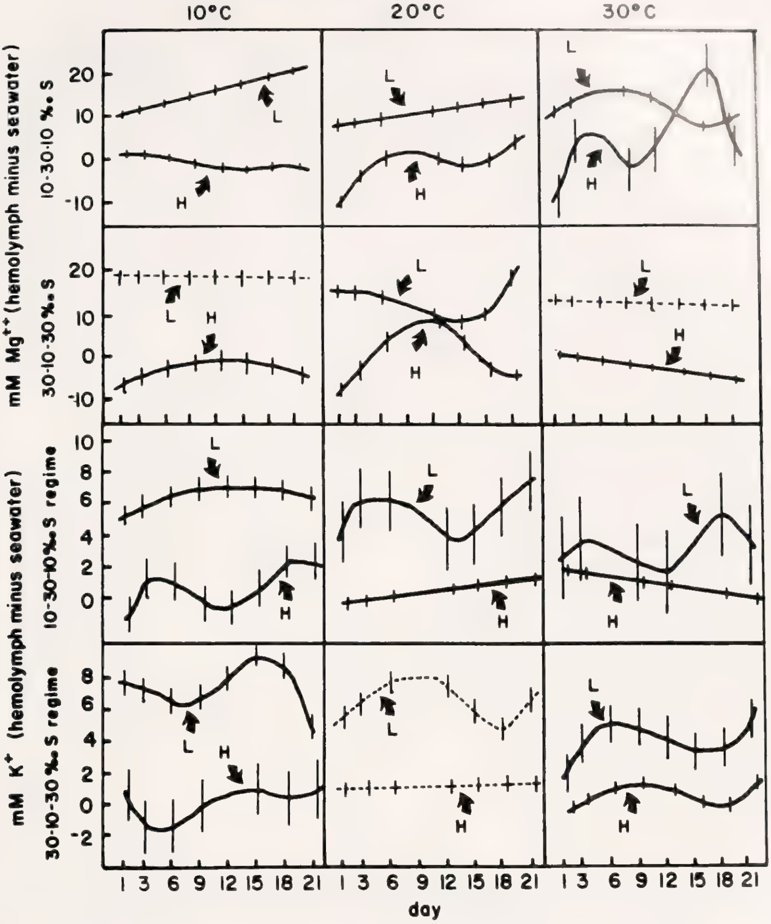


FIGURE 3. Regression lines for hemolymph Mg^{2+} and K^+ concentrations of *Thais haemastoma* expressed as hemolymph-minus-seawater concentration at the high (H) and low (L) phases of salinity cycles. Lines are presented for 10–30–10‰ and 30–10–30‰ S cycles at 10°, 20°, and 30°C. Vertical lines represent standard error at each sample point.

although no interactions were significant. Duncan's test showed hemolymph minus seawater [K^+] was greatest at 10°C and least at 30°C.

Ninhydrin-positive substances

Ninhydrin-positive-substances (NPS) concentration of the foot, calculated as μM NPS/g dry weight and as μM NPS/g tissue water, are presented in Table II. Data are shown for 15 steady-state temperature-salinity combinations. NPS content of the foot expressed as either NPS/g dry weight or NPS/g tissue water increases with increasing salinity at all three temperatures studied. NPS content of the foot tends to be lower at 10°C than at the other temperatures. No other clear trends are evident.

NPS/g dry weights were not significantly different from control (day 0) values at high- and low-salinity sample points on the first day of salinity fluctuations (Fig. 4). Snails acclimated to 30‰ S had higher NPS levels in the foot even at

TABLE II

Ninhydrin positive substances (NPS) in the foot of Thais haemastoma *acclimated to 15 temperature-salinity combinations expressed as both μM NPS/g dry weight and as μM NPS/g tissue water. Values given are $\bar{X}$ ± 95% confidence limit (n). ND indicates no data available.*

	7.5‰	10‰	20‰	30‰	35‰
10°C					
μM/g dry weight	87.0 ± 19.4 (6)	144.7 ± 3.3 (5)	183.6 ± 10.5 (4)	268.2 ± 31.6 (4)	ND
μM/g tissue H_2O	23.6 ± 4.0 (6)	40.9 ± 8.0 (4)	94.3 ± 7.6 (4)	94.3 ± 7.6 (4)	ND
20°C					
μM/g dry weight	124.3 ± 1.7 (5)	129.0 ± 14.5 (5)	211.0 ± 7.3 (5)	312.0 ± 34.5 (4)	338.1 ± 25.3 (1)
μM/g tissue H_2O	37.3 ± 2.2 (5)	41.0 ± 5.2 (5)	66.8 ± 9.4 (4)	96.5 ± 18.7 (4)	117.37 ± 10.4 (4)
30°C					
μM/g dry weight	115.4 ± 3.4 (4)	159.9 ± 9.9 (5)	217.64 ± 45.4 (3)	244.6 ± 51.3 (3)	241.19 ± 24.7 (3)
μM/g tissue H_2O	34.4 ± 3.2 (4)	48.7 ± 4.5 (4)	68.1 ± 4.2 (3)	93.6 ± 5.8 (3)	84.9 ± 2.0 (3)

corresponding points of the salinity fluctuation cycle, that is, when both were measured at 10 or 30‰ S. NPS/g dry weight of snails acclimated to 30‰ and subjected to salinity cycles of 30–10–30‰ S did not change significantly over the 3-week experiment at 10°, 20°, or 30°C. In contrast, snails acclimated to 10‰ S and subjected to salinity cycles of 10–30–10‰ exhibited significantly higher NPS/g dry weight values than control and day 1 values after 2 weeks and remained higher after 3 weeks of cycling salinity. There was little significant cycling of NPS levels, expressed as μM NPS/g dry weight, with the daily salinity fluctuations. Similar trends were observed when these data were analyzed expressing NPS levels as μM NPS/g tissue water except that NPS levels cycled with fluctuating salinity to a greater extent, tending to be higher during high salinity slackwater sample periods (Fig. 4).

DISCUSSION

Although little difference existed between hemolymph and seawater osmolality or ion concentrations in *Thais haemastoma* at constant salinities and temperatures studied, temperature affected hemolymph values over a range of salinities. The differences between hemolymph and seawater osmolality and Cl^- and Na^+ concentrations were greatest at 10°C. Mg^{2+} hemolymph-minus-seawater concentration was less at 10° and 30°C than at 20°C, while hemolymph K^+ levels were not affected by temperature under steady-state conditions. Pierce (1970) argued that the slight hyperosmotic concentrations of blood and pericardial fluids relative to sea water in *Modiolus* was due to a Gibbs-Donnan equilibrium. However, Mangum and Johansen (1975) have shown that a Gibbs-Donnan equilibrium is not responsible for the frequently observed hyperosmoticity of body fluids relative to ambient water in osmoconforming marine invertebrates. Therefore, mechanisms responsible for *T. haemastoma* maintenance of hemolymph osmolality, Na^+, Mg^{2+}, K^+, and Cl^- levels at concentrations significantly different from that of the ambient water remain to be elucidated.

When salinity was cycled, hemolymph osomolality and ion concentrations were maintained above seawater values during the low phase of salinity cycles and were usually isosmotic during the high phase at 10°, 20°, and 30°C. Less cycling of hemolymph ion concentrations with salinity cycles occurred at 10°C than at 30°C, especially of hemolymph Mg^{2+} and K^+ concentrations, but there were still significant hemolymph changes during most cycles. Similar results have been reported for

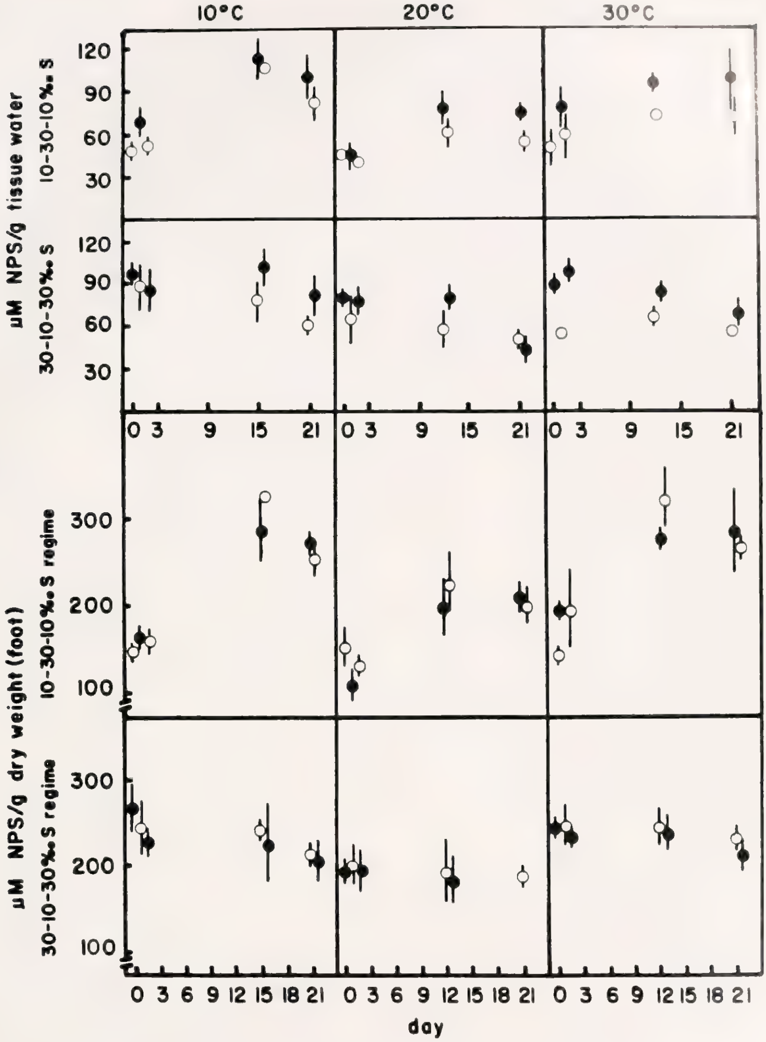


FIGURE 4. *Thais haemastoma.* Mean plus or minus the 95% confidence limits are given for NPS/g dry weight and NPS/g tissue water in the foot of *Thais haemastoma* at the high and low phase of salinity cycles. Data are given for 10–30–10‰ and 30–10–30‰ S cycles at 10°, 20°, and 30°C. Open circles represent high salinity sample points; closed circles, low salinity points. Lack of error bars indicates confidence limits smaller than the point on the figure.

T. haemastoma exposed to 14 days of a 20–10–20‰ S diurnal pattern of fluctuating salinity at 15°–21°C. (Stickle and Howey, 1975).

The observed hemolymph-to-seawater ion differences during salinity fluctuation in *Thais haemastoma* are probably passive. Hemolymph was isionic during the high phase of salinity cycles and hyperionic during the low phase, suggesting greater solute and solvent movement during periods of increasing salinity. Extracellular fluid of other osmoconforming molluscs (Stickle and Ahokas, 1975; Stickle and Howey, 1975; Hand and Stickle, 1977, Shumway, 1977) and echinoderms (Stickle and Ahokas, 1974) may also remain hypersomotic during the low phase of salinity cycles. If the hemolymph-to-seawater difference in ion concentration during the low phase of salinity cycles at 10°C were caused by metabolic-dependent activities, regulation would have been observed under steady-state conditions also. Magnesium was the only hemolymph ion maintained at levels significantly different from seawater values under steady-state conditions. All other hemolymph ions were not different from seawater values even after 5 weeks acclimation to 10°C, suggesting low-temperature changes in membrane permeability or changes in other passive activities during salinity fluctuations. Prusch and Hall (1978) found that *Mytilus edulis* is capable of altering tissue water permeability with changes in osmolality of the ambient water. Similar permeability changes may be occurring in response to temperature and fluctuating salinity in *T. haemastoma*. Oxygen consumption in *T. haemastoma* exposed to single salinity cycles at 20°C decreases in the middle of 10–15–10‰, 30–10–30‰ and 10–30–10‰ S diurnal cycles (Findley *et al.*, 1978), suggesting a passive mechanism is responsible for the differences between hemolymph and seawater during the low phase of salinity cycles.

Thais haemastoma maintains higher hemolymph osmolality at lower temperatures under both steady-state and fluctuating salinity conditions. Hemolymph osmolality and ion concentrations are regulated above seawater values during the low phase of salinity cycles to a much greater extent at 10°C than at 30°C. Thus it seems *T. haemastoma* is better able to withstand dilute and widely fluctuating salinities at low temperatures. Lowest field salinities in Barataria Bay, the collection site for experimental animals, occur during winter (Hewatt, 1951). *T. haemastoma* may be able to compensate for low environmental salinities by passively regulating hemolymph composition when natural tidal salinity fluctuations occur most frequently. Since the feeding threshold of *T. haemastoma* is about 12.5°C (Garton and Stickle, 1980) it cannot be concluded that *T. haemastoma* would be able to permanently colonize dilute waters at cold temperatures. Indeed it is generally considered to be a warm water species extending only as far north as North Carolina.

Ninhydrin-positive substances (NPS) increased in foot tissue of *T. haemastoma* with increasing salinity and were lower at 10°C than at other experimental temperatures under steady-state salinity conditions. Ninhydrin-positive substances are principally composed of free amino acids which are important in cell volume regulation in response to salinity variation. Peterson and Duerr (1969) found the free amino acid levels of *Tegula funebralis* maintained for 11 days at 50, 100, 120, and 160% seawater (35‰ S = 100%) to increase linearly from 50 to 120% seawater and then to decline to approximately 100% seawater concentration at 160% seawater when based on grams/wet-weight. *Modiolus* hearts (Pierce and Greenberg, 1973) and intact *Mytilus edulis* (Livingstone *et al.*, 1979) transferred directly from high to low salinity release free amino acids from the intracellular fluid compartment. Free amino acids increase in acclimation of bivalve molluscs to high salinity (Baginski and Pierce, 1977, Gainey, 1978). Expressing NPS concentration in the foot of *T. haemastoma* on a dry weight basis indicates synthesis or degradation of NPS, principally free amino acids, whereas expressing NPS concentration on a tissue water basis provides information on the osmotic effectiveness of free amino acids. Extracellular fluid concentrations of NPS are small in comparison with entire foot NPS, suggesting that most NPS are intracellular. Stickle and Howey (1975) found hemolymph NPS concentrations to range from 0.6 to 6.4 mM in snails acclimated to 10–30‰ S. Foot tissue water NPS concentrations range from 41 to 117 mM between 10 and 30‰ S.

Variation in the volume of the intracellular fluid compartment without a concomitant change in absolute NPS content will alter the osmotic importance of NPS. Staaland (1970) found the intracellular fluid compartment of *Buccinum undatum* to vary inversely with salinity between 10 and 33‰ whereas no change occurred in the size of the extracellular fluid compartment. If a similar relationship exists in the foot of *T. haemastoma* the osmotic effectiveness of NPS as intracellular osmotically active particles will be further reduced by dilution of the intracellular fluid compartment at low salinity.

Under fluctuating salinity conditions, it was more difficult to obtain hemolymph at 10°C than at 20°C while hemolymph was most abundant at 30°C. Stickle and Howey (1975) found similar results with *T. haemastoma* exposed to two weeks of diurnal 20–10–20‰ patterns of fluctuating salinity. Total body water in the foot of stepwise acclimated *T. haemastoma* was not significantly different between 10° and 30°C, which suggests that the intracellular fluid compartment was probably larger at 10°C than at 20° or 30°C.

Ninhydrin positive substances did not cycle in the foot of *T. haemastoma* in response to cycling salinities, although NPS levels increased with time in snails acclimated to 10‰ S and subjected to a long-term 10–30–10‰ regime. In contrast, all eight bivalve species studied by Shumway *et al.* (1977) cycled adductor muscle NPS levels in response to 1-day salinity fluctuations. After 1 week of salinity fluctuation, no changes in *Mytilus edulis* tissue NPS levels were observed during a salinity cycle. Additionally, tissue NPS did not decline below the concentration observed in 100% seawater. On the other hand, Livingstone *et al.*, (1979) found changes in *M. edulis* NPS level to be small during short-term salinity fluctuations (30–15–30‰ cycles) but to decline markedly after exposure to 24 days of salinity fluctuations. These authors believe that the lack of NPS decline during fluctuating salinity observed in the study of Shumway *et al.*, (1977) may have been due to a combination of valve closure during the low-salinity phase of each cycle and salinity effects. The mussels used by Livingstone *et al.*, (1979) remained open throughout salinity cycles and continued to respire. Likewise, the *T. haemastoma* used in our fluctuating-salinity experiments remained attached to aquarium walls and crawled and preyed on oysters, although less activity was observed at 10°C than at other temperatures studied. Garton and Stickle (1980) have documented the feeding activity of oyster drills on oysters under identical conditions and Findley *et al.*, (1978) have shown aerobic respiration to occur under fluctuating salinity conditions.

Thais haemastoma passively regulates hemolymph osmolality and ionic composition slightly above environmental levels at low temperatures and constant salinity, as well as during the daily low phase of long-term salinity fluctuation cycles. Since cell fluid volume appears to be greater at low temperatures and low salinities, NPS may be less important in osmotic regulation and cell volume regulation under conditions of low environmental temperature and salinity.

This research was partially supported by a PRECOL Research Fellowship to J.E.H. during the summer and was done as partial fulfillment of the requirements for the Master of Science Degree. Tom Sabourin, Dave Garton, Preston Newman and Barbara Belisle provided technical assistance during this project. The authors wish to acknowledge with thanks the critical review of this manuscript offered by Dr. Thomas Dietz, Dr. John Fleeger, Dr. Earl Weidner, and Mr. David Saintsing.

SUMMARY

1. When *Thais haemastoma* were acclimated stepwise to constant salinity; hemolymph osmolality, Na^+, Mg^{2+}, K^+, and Cl^- concentrations as well as tissue NPS concentration increased with salinity between 7.5 and 30‰ at 10°, 20°, and 30°C. Hemolymph osmolality was significantly greater than that of seawater at 10, 20, 30‰ S and 10°C. Per cent tissue water was unaffected by temperature and decreased with increasing salinity and hemolymph osmolality. The hemolymph concentration of all ions increased with increasing concentrations in seawater. Hemolymph $[\mathrm{Cl}^-]$ was lower than in seawater at 20° and 30°C but hyperionic to seawater at 20 and 30‰ S and 10°C. Hemolymph $[\mathrm{Na}^+]$ was greater than in seawater at 10°C but no difference existed at 20° and 30°C. Hemolymph $[\mathrm{Mg}^{2+}]$ was significantly greater than in seawater at 10 and 20‰ S at all temperatures. Neither temperature nor salinity significantly affected the hemolymph-to-seawater difference in $[\mathrm{K}^+]$. [NPS] in the foot of *T. haemastoma* increased with salinity and was lower at 10°C than at 20° and 30°C.

2. Hemolymph osmolality tracked seawater osmolality less well at 10°C than at 20° and 30° during 30–10–30‰ and 10–30–10‰ diurnal patterns of fluctuating salinity. Reduced tracking at 10°C was due to less decline of hemolymph osmolality during the decreasing-salinity phase of the cycles. Snail hemolymph osmolality also declined less during the decreasing-salinity phase of the 30–10–30‰ S cycle than during the 10–30–10‰ S cycles, indicating that acclimation to different constant salinities prior to the initiation of fluctuating salinity cycles has a significant effect on the response of *T. haemastoma* to fluctuating salinity. Tissue water in the foot varied inversely with seawater and hemolymph osmolality during fluctuating salinity. The difference in percent tissue water between snails acclimated to 10 and 30‰ S prior to salinity fluctuation was greatest at 30°C, intermediate at 20°C, and least at 10°C. Hemolymph $[\mathrm{Na}^+]$, $[\mathrm{Mg}^{2+}]$, $[\mathrm{K}^+]$, and $[\mathrm{Cl}^-]$, tracked ambient salinity least well at 10°C and most closely at 30°C. Passive factors are likely to be responsible for the differences in hemolymph osmotic and ionic fluctuation observed at 10°, 20°, and 30°C. Tissue NPS did not fluctuate with daily salinity cycles in either the 30–10–30‰ or 10–30–10‰ diurnal patterns of fluctuating salinity. Foot tissue NPS of snails acclimated to 30‰ did not change over three weeks of exposure to a 30–10–30‰ S pattern of fluctuating salinity but NPS in the foot of snails acclimated to 10‰ S and subjected to a 10–30–10‰ S pattern of fluctuating salinity increased significantly in 15 days and remained significantly higher on Day 21.

LITERATURE CITED

BAGINSKI, R. A., AND S. K. PIERCE, 1977. The time course of intracellular free amino acid accumulation in tissues of *Modiolus demissus* during high salinity adaptaton. *Comp. Biochem. Physiol.*, 57: 407–412.

BARR, A. J., J. H. GOODKNIGHT, J. P. SALL, AND J. T. HELWIG, 1976. *A User's Guide to SAS 76.* Raleigh, North Carolina: SAS Institute Inc.

DEHNEL, P. A., 1962. Aspects of osmoregulation in two species of intertidal crabs. *Biol. Bull.*, 122: 208–227.

DEHNEL, P. A., AND T. H. CAREFOOT, 1965. Ion regulation in two species of intertidal crabs. *Comp. Biochem. Physiol.*, 15: 377–397.

Findley, A. M., B. W. Belisle, and W. B. Stickle, 1978. Effects of salinity fluctuations on the respiration rate of the Southern Oyster Drill *Thais haemastoma* and the Blue Crab *Callinectes sapidus*. *Mar. Biol.*, **49**: 59–67.

Gainey, L. F., Jr., 1978. The response of the Corbiculidae (Mollusca: Bivalvia) to osmotic stress: the cellular response. *Physiol. Zool.*, **51**: 79–91.

Garton, D., and W. B. Stickle, 1980. Effects of salinity and temperature on the predation rate of *Thais haemastoma* on *Crassostrea virginica* spat. *Biol. Bull.*, **158**: 49–57.

Hand, S. C., and W. B. Stickle, 1977. Effects of tidal fluctuations of salinity on the pericardial fluid composition of the American Oyster *Crassostrea virginica*. *Mar. Biol.*, **33**: 309–322.

Hewatt, W. G., 1951. *Salinity studies in Louisiana coastal embayments west of the Mississippi River.* 32 pp. Final Report of Project Nine. Texas A & M. Research Foundation College Station, Texas.

Kinne, O., 1970. Temperature: Invertebrates. Pp. 407–514 in O. Kinne, Ed., *Marine Ecology, Vol. 1. Environmental Factors, Part 1.* London: Wiley-Interscience.

Kinne, O., 1971. Salinity: Invertebrates. Pp. 821–996 in O. Kinne, Ed., *Marine Ecology Vol. 1. Environmental Factors, Part II.* London: Wiley-Interscience.

Livingstone, D. R., J. Widdows, and P. Fieth, 1979. Aspects of nitrogen metabolism of the common mussel *Mytilus edulis:* adaptation to abrupt and fluctuating changes in salinity. *Mar. Biol.*, **53**: 41–56.

Lockwood, A. P. M., 1960. Some effects of temperature and salinity of the medium on the ionic regulation of the isopod *Asellus aquaticus* (L.). *J. Exp. Biol.*, **37**: 614–630.

Mangum, C. P., and K. Johansen, 1975. The colloid osmotic pressures of invertebrate body fluids. *J. Exp. Biol.*, **63**: 661–671.

Peterson, M. B., and T. G. Duerr, 1969. Studies on osmotic adjustment in *Tegula funebralis* (Adams, 1954). *Comp. Biochem. Physiol.*, **28**: 633–644.

Pierce, S. K., Jr., 1970. The water balance of *Modiolus* (Mollusca: Bivalvia): osmotic concentrations in changing salinities. *Comp. Biochem. Physiol.*, **36**: 521–533.

Pierce, S. K., and M. J. Greenberg, 1973. The initiation and control of free amino acid regulation of cell volume in salinity stressed marine bivalves. *J. Exp. Biol.*, **59**: 435–440.

Prusch, R. D., and C. Hall, 1978. Diffusional water permeability in selected marine bivalves. *Biol. Bull.*, **154**: 292–301.

Rosen, H., 1957. A modified ninhydrin colorimetric analysis for amino acids. *Arch. Biochem. Biophys.*, **67**: 10–15.

Shumway, S. E., 1977. The effect of fluctuating salinity on the tissue water content of eight species of bivalve molluscs. *J. Comp. Physiol.*, **116**: 269–285.

Shumway, S. E., P. A. Gabbot, and A. Youngson, 1977. The effect of fluctuating salinity on the concentrations of free amino acids and ninhydrin positive substances in the adductor muscles of eight species of bivalve molluscs. *J. Exp. Mar. Biol. Ecol.*, **29**: 131–151.

Staaland, H., 1970. Volume regulation in the common whelk, *Buccimun undatum* L. *Comp. Biochem. Physiol.*, **34**: 355–365.

Stickle, W. B., and R. Ahokas, 1974. The effects of tidal fluctuation of salinity on the pericardial fluid composition of several echinoderms. *Comp. Biochem. Physiol.*, **47**: 469–476.

Stickle, W. B., and R. Ahokas, 1975. The effects of tidal fluctuation of salinity on the hemolymph composition of several molluscs. *Comp. Biochem. Physiol.*, **50**: 291–296.

Stickle, W. B., and T. W. Howey, 1975. Effects of tidal fluctuations of salinity on hemolymph composition of the Southern Oyster Drill *Thais haemastoma*. *Mar. Biol.*, **33**: 309–322.

Taylor, E. W., P. J. Butler, and A. Al-Wassia, 1977. The effect of a decrease in salinity on respiration, osmoregulation, and activity in the shore crab, *Carcinus maenus* (L.) at different acclimation temperatures. *J. Comp. Physiol.*, **119**: 155–170.

Todd, M. E., 1963. Osmoregulation in *Ligea oceanica* and *Iodotea granulosa*. *J. Exp. Biol.*, **40**: 381–392.

Verway, J., 1957. A plea for the study of temperature influence on osmotic regulation. *Annee Biol. 3 serie*, **33**: 129–149.

Reference: *Biol. Bull.*, **159**: 162–176. (August, 1980)

DENSITY-DEPENDENT GROWTH INHIBITION IN LOBSTERS, *HOMARUS* (DECAPODA, NEPHROPIDAE)[1]

KEITH NELSON, DENNIS HEDGECOCK, WILL BORGESON, ERIC JOHNSON, RICHARD DAGGETT, AND DIANE ARONSTEIN

University of California, Bodega Marine Laboratory,
Bodega Bay, California 94923

Delay of molt of juvenile lobsters (*Homarus americanus*) by earlier-molting neighbors has been demonstrated by Cobb and Tamm (Cobb, 1968, 1970; Cobb and Tamm, 1974, 1975a–c). Studies in our laboratory have indicated density-dependent inhibition, including a "nearest-neighbor" effect, upon lobster growth (Hedgecock *et al.*, 1976; Hedgecock and Nelson, 1978; Hand *et al.*, 1977). In contrast to our results, Cobb and Tamm (1975a) concluded that physical contact was necessary to produce the molt delay they observed; visual and/or chemical contact were insufficient. Such effects are of potentially great importance to crustacean aquaculture and under certain conditions may play a role in regulating growth in nature. The present paper describes an experiment with juvenile lobsters that allowed discrimination among several categories of chemically mediated growth inhibition and demonstrated a powerful short-range effect.

MATERIALS AND METHODS

System design

Two semi-recirculating seawater systems, I and II, were used to culture individually-held juveniles. The systems are similar to that illustrated in Figure 2 of Hand *et al.* (1977) but include several modifications important for the study of chemical effects of animal density (Fig. 1): a) Makeup water is first treated to remove supersaturated gases. b) Returning water overflows via an adjustable standpipe at a rate equal to the makeup rate. The remainder mixes with the makeup water in sump compartment S′ where gas pressures and temperature may be regulated. Separation of sump function into two compartments offers accurate control of the makeup fraction. c) Flow through the culture tray is channeled in eight columns, each isolated by solid partitions, and each consisting of 15 compartments separated by perforated partitions. Three trays are connected in series but with intervening mixing troughs and siphon manifolds to prevent differences between adjacent columns from accumulating from one tray to the next. d) Compartment volume may be adjusted via a dam following the last mixing trough. The table design allows experimental study of changes in concentration of water-borne density-dependent factors without varying the space allotted to each animal. The gradient system of Cobb and Tamm (1974) did not allow analysis by a compartmental model (see compartmental analyses below), but was otherwise similar.

---

[1] This work is a result of research sponsored by NOAA Office of Sea Grant, Department of Commerce, under Grant No. 04–8–MO1–189.

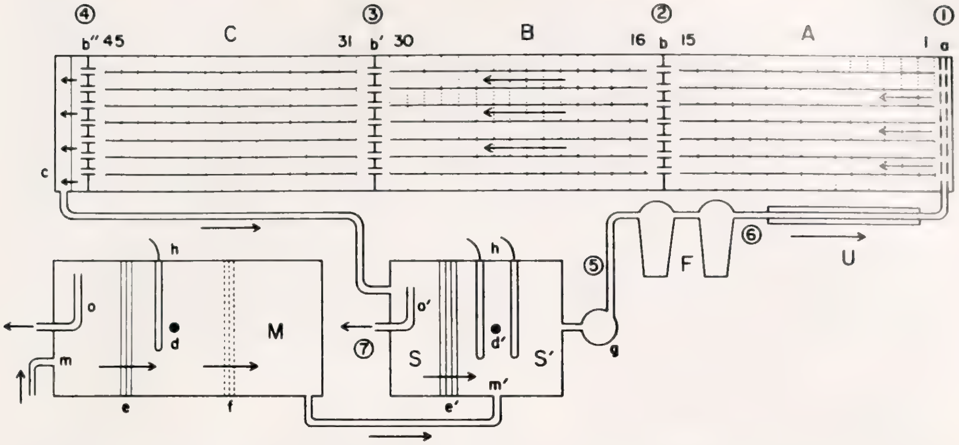


FIGURE 1. Semi-recirculating seawater system. A, B, C, culture trays; F, 25 and 5 μ canister filters; M, makeup water treatment box; S, S′, compartments of sump; U, 50 W UV water treatment unit. Details are: a, 4 l mixing trough with perforated distributor; b, b′, b″, pairs of 4 l mixing troughs joined by siphon manifolds; c, adjustable dam and return line; d, d′, airstones; e, e′, baffles; f, degassing screens; g, pump; h, heaters; m, untreated, and m′, treated makeup water input; o, o′, overflow standpipes. Circled numbers are sampling points for water quality analyses; uncircled numbers indicate rows.

Experimental subjects and protocol

Two age classes of juveniles (herein called older and younger) were used. "Older" individuals were hatched from three wild-caught *Homarus americanus* females, reared in larval systems (Hand *et al.*, 1977) until metamorphosis, and then maintained in individual compartments at ambient temperatures (*ca.* 14°C) until the beginning of the experiment, at which time virtually all were nearing the 5th-to-6th-stage molt. On Julian Calendar Day 288/78, equal numbers from each of the three females (288 in all) were randomly assigned to the spaces in rows 5–10 of each tray (numbering from the influent end). "Younger" juveniles, from a single laboratory mating of a male *H. americanus* to a female *H. gammarus*, were hatched on Day 270/78 ± 3 days and reared at 19°C until Day 288/78, at which time all were in the 4th (1st post-larval) stage. (Pure *H. americanus* individuals of appropriate age and numbers were unavailable. We are confident, however, that using hybrids did not materially affect the results reported.) A total of 432 "younger" individuals were then randomly assigned to the remaining nine rows in each tray. Backup animals for each family were maintained in another semi-recirculating seawater table at 21°C.

Within the first 7 days, 37 lobsterlings died in System I, and 55 in System II. Deaths thereafter were negligible except in rows B 10–12 of System II, where warping of the plastic tray bottom allowed cannibalization of a few molted animals. All dead lobsters were replaced and data from the replacements were used in the analysis.

Throughout most of the experiment animals were maintained on a 7 hr light : 17 hr dark photoperiod, with an additional ½ hr of light approximately 6 hr into the dark period for a nightly check and molt collection. Lighting was from four standard 40 W clear white fluorescent tubes fixed 1.5 m above the culture trays and covered with two layers of green cellophane to reduce algal growth. Molted carapaces were collected twice daily and their lengths recorded when possible, before they were returned to the proper compartments. Animals were fed once daily *ad libitum* with adult brine shrimp and compartments were siphoned daily before feeding. Mixing troughs and sump compartments were siphoned as needed. Cartridge filters were replaced weekly.

The experiment was terminated after 93 days, at which time weights and carapace lengths of all animals were recorded. The hybrid animals at this time were approximately 111 days old and the specimens of *H. americanus* approximately 1 month older. Only carapace length (measured from the rear edge of the orbit) and corresponding results will be reported in this paper since weight results are highly correlated with length results (Hedgecock *et al.*, 1976; Hedgecock and Nelson, 1978).

Chemical and physical analyses

Temperature, recorded thrice daily, was maintained at 21° and 20.5°C, respectively, at points 1 and 4 (see Fig. 1) with little variation between systems or from day to day. Dissolved oxygen, pH, and ammonia were measured daily at points 1 and 4, and weekly at points 2, 3, 5, and 6. Nitrate, nitrite, and organic nitrogen were analyzed weekly at sample locations 1–6. Methods of analysis for nitrogen compounds are given in Daggett and Aronstein (1979). Non-filterable residue > 0.45 μm was measured weekly at points 1, 4, and 5. Bacterial counts were made according to procedures in Standard Methods (1976) on samples taken weekly from points 1 and 4.

The systems were designed to create gradients of long-lived metabolite concentration in each system, from the influent mixing trough (sampling point 1, Fig. 1) to the effluent mixing trough (sampling point 4). By using different make-up rates but the same table flow rate (2 l/min) in the two systems, the fraction of new, non-recirculated sea water entering the table was controlled so that the gradient of long-lived metabolite concentration in System II, while no steeper, began at a threefold higher concentration. For this, the fraction of the water entering the table that was new make-up water was maintained at 50% in System I and 25% in System II. These fractions were used so that the concentrations of long-lived metabolites such as ammonia at points 1 and 4 in System I and points 1 and 4 in System II would be maintained in the approximate proportions 1 : 2 : 3 :4, respectively, assuming equal *rates* of metabolite buildup in the two systems but irrespective of their actual magnitude (see compartmental analyses below). Terminal gradients from point 1 to point 4 in both systems were 50–75 μg nitrogen/l, occasionally higher. Concentrations of ammonia in System II were always considerably higher than in System I, as anticipated; the animals in the final rows of System II were occasionally exposed to concentrations greater than 500 μg nitrogen/l ($NH_3 + NH_4^+$). These levels are nevertheless very much lower than the "safe" levels suggested by Delistrary *et al.* (1977) for 4th-stage lobsters. Sampling at points 5 and 6 showed a small (< 10 μg nitrogen/l), inconsistently positive or negative effect on ammonia concentration by cartridge filtration, and a slight positive effect (< 2 μg/l) of UV treatment.

Oxygen concentration, restored to ambient values by aeration in the sump, was used as a model of the behavior of concentration of long-lived metabolites removed by filtration or aeration (see compartmental analyses below). Oxygen concentration gradients (ΔO_2) from points 1 to 4 were similar in the two systems. Only once, following pump failure, was a ΔO_2 steeper than -2 mg O_2 l observed. Terminal ΔO_2's averaged about -1 mg O_2 l. Weekly samples at points 1, 5, and 6 were near saturation and the gradient across points 1–4 was linear. A small negative gradient in pH was maintained in both systems (minimum -0.2 units, from 8.1 to 7.9 at points 1 and 4, respectively; generally much closer to zero). NO_2, NO_3, organic nitrogen, and non-filterable residue concentrations displayed no consistent gradient in either system, and very small differences from ambient values.

Bacterial counts' gradients were similar in the two systems, with values of <10/ml at point 1 and highly variable values at point 4. The means of the natural logarithms of the counts at point 4 on Tables I and II, respectively, were 7.886 ± 0.416 (s.e.) and 7.979 ± 0.518. There was no evident correlation of bacterial counts with other water quality variables, except for a general increase with time.

Following the experiment, logs of the absorbances of water samples taken 1, 2, 4, 8, and 16 min after methylene blue injection into 10 individual compartments were separately regressed on time and the slopes averaged to obtain an estimate of the first-order compartment-turnover rate, $k = 0.631 \pm 0.033$ per min. As the average compartment contained 0.25 l and the compartment flow rate was 0.25 l per min, the expected k is 1.0 per min, and mixing was thus not complete. The typical flow pattern within a compartment contained one or two gyres with vertical axes. Nevertheless, we will use a linear model with $k = 0.631$ per min in the ensuing analyses (see compartmental analyses below). In this dye test, virtually no color was observed to flow into neighboring upstream compartments, although this type of flow could have occurred during the experiment proper.

Experimental design

The block of older animals in the middle of each tray necessitates treating the design as three separate experiments (2 systems × 3 trays × k rows, with k equal to 4, 6, and 5, respectively, for the first 4 rows of younger animals, the next 6 rows of older individuals, and the last 5 rows of younger animals). While physically the experimental design is fully nested, the experimental variable is *position* (along the gradient from point 1 in System I to point 4 in System II); and we are interested in separately examining the interactions among system, tray, and row. Therefore, we treat the experiment as three three-factor, fixed-effect, factorial designs with eight replicates (Table I).

Compartmental analyses

A simple generalized catenary compartmental model (Atkins, 1969) for the concentration $a_j(t)$ of a particular metabolite in the j-th compartment at time t will be of use in discussing both the performance of the system and the growth difference results:

$$\frac{d(a_j)}{dt} = \frac{r_j}{v_j} + k_{ji}\frac{v_i}{v_j}a_i - k_{kj}a_j - k_{0j}a_j, \quad \begin{array}{l} i = j - 1 \\ j = 1, 2, 3 \ldots \\ k = j + 1 \end{array} \qquad (1)$$

where r_j is the rate of production (consumption, removal) of substance in units of mass per unit time, v_j is the volume in liters of compartment j, k_{ji} is the turnover rate (rate constant) from the preceding compartment i, and k_{0j} represents the rate constant of loss (or gain, $+ k_{jo}a_o$) of the substance from the compartment to (or from) outside the system; all k's are in units of reciprocal time. For our purposes all k's will be assumed to be constants, although k_{0j} may be a function of the difference between a_j and a_o, the concentration outside the system.

In the case of oxygen utilization the r_j's are negative and if we assume no addition to the compartment at the air-water interface k_{j0} is zero and the right-hand term drops out. If we assume the v's and k's to be equal and that the water suffers no change in the mixing troughs, equation (1) has the steady-state solution

$$a_j = a_0 \quad \sum_{l=1}^{j} \frac{r_l}{vk} \tag{2}$$

with a_0 the ambient concentration of oxygen, $v = 0.25$ l, and k experimentally determined as 0.631 per minute. The average weight of a lobster near the end of the experiment was somewhat less than 2 g. Assuming a 2 g weight, the oxygen concentration gradient ΔO_2 from sampling point 1 to sampling point 4 (*i.e.*, a_{45} minus a_0) becomes -1.5 mg O_2/l, using the O_2 consumption value of 5.25 μg per lobster per min obtained from the regression of Logan and Epifanio (1978). Our experimental values are very close to this. The gradients in O_2 concentration were approximately linear; given the flow rates and surface–volume relationships involved, it seems safe to assume that the rate constant k_{oj} or k_{jo} for loss or gain of "volatiles" at the air-water interface were not large compared with the k_{ji}'s.

For ammonia ($NH_3 + NH_4^+$) concentration, assuming that there is loss only at the overflow o′ and that makeup water concentration is negliglible, the steady state equation (2) becomes

$$a_j = (R - 1) \sum_{l=1}^{45} \frac{r_l}{vk} + \sum_{l=1}^{j} \frac{r_l}{vk}. \tag{3}$$

R, the recirculation multiplier, is the ratio of the flow rate through the table to the makeup flow rate into the sump, *i.e.*, the reciprocal of the makeup fraction. (This R is not to be confused with the *R* in a similar model of Liao and Mayo, 1972, which they define as "% of water reused." See also Delistrary *et al.*, 1977.) Using an ($NH_3 + NH_4^+$) production value of 0.4 mg per lobster per day for a 2-g lobster (quoted by Moffett and Fisher, 1978, from unpublished work) we obtain a gradient from point 1 to point 4 of 79 μg/l, again consistent with our observed values. With the makeup fractions used, of course, equation (3) predicts the 1:2:3:4 ratio for a_0 and a_{45} in the two systems.

Considering only the downstream effect of a short-lived inhibitor, the appropriate compartmental model is equation (1) with r_j's greater for the older than for the younger animals (because of their larger size). We are interested only in the difference between the older and younger animals' rates of production, and therefore for simplicity we will consider the hypothetical growth-inhibiting substance as originating exclusively with the older ones and only in the last row of their block. Then the steady-state solution for the compartments downstream from the block is

$$a_j = \frac{k_2^{j-1} r_1}{v(k_1 + k_2)^j}, \quad j = 1, 2, 3 \ldots \tag{4}$$

where k_1 is the rate of loss or decay of the substance, k_2 is the turnover rate to the next compartment, r_1 is the excess production rate by the older animals and v is the compartment volume.

For $k_1 = k_2 = 0.631/\text{min}$ and $r_1/v = 1$ g per l per min we have plotted the expected steady-state concentrations (a_j) in the rows downstream from the older animal row ("row 1") in Figure 4B.

The downstream effect of pulsed production of an inhibitory substance, with the pulses far apart in time, may be simulated by equation (1) with the first and last terms eliminated, and with a unit impulse function as initial condition. There are repeated roots and the solutions are of the form

$$a_j(t) = A_j t^{j-1} e^{-kt} \qquad (5)$$

With compartment sizes and turnover rates assumed to be equal in the different compartments, the time integrals of concentration in the different compartments will be the same. One may thus equate the time integrals of the solutions (5) for each j and solve for the A_j's in terms of the initial concentration pulse A_0. Equation (5) then becomes

$$a_j(t) = \frac{k^{j-1}}{(j-1)!} A_0 t^{j-1} e^{-kt} \qquad (6)$$

which for $A_0 = 1$ g/l and $k = 0.631/\text{min}$ is plotted in Figure 4A.

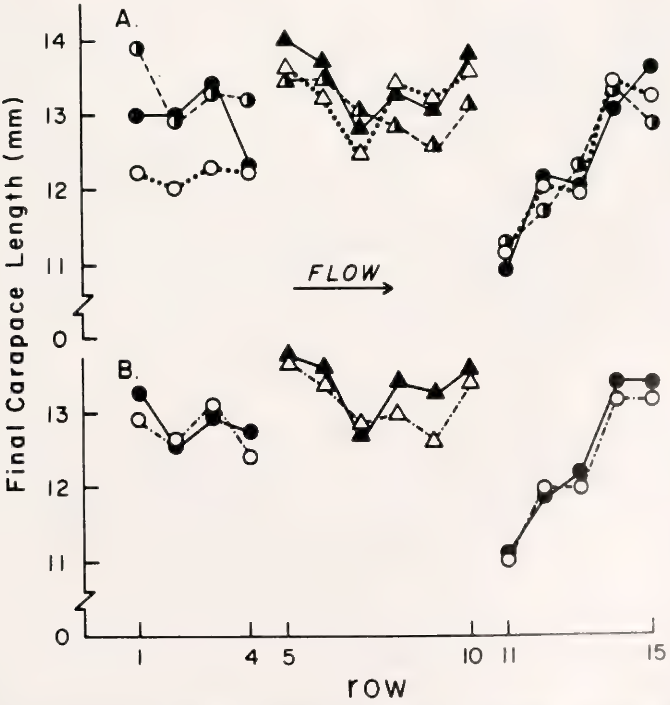


FIGURE 2. Average final carapace lengths of animals (ordinates) plotted against position in a tray (abscissa). Rows 5–10 contain the older *Homarus americanus* lobsters. In A, data from corresponding rows of the two systems are pooled. Open circles, trays A; half-filled circles, trays B; filled circles, trays C. In B, data from corresponding rows in trays A, B, and C are pooled. Open circles, System I; closed circles, System II.

Results

Final carapace length difference

Table I gives results of ANOVAs for the three "experiments." Factor 1 (System) never reaches a significant F-ratio. Figure 2B displays graphically the mean carapace–length values for each row in each system, lumping over trays; it may be seen that there is indeed no difference between Systems I and II in this respect.

Factor 2 (tray position) reaches significance ($P < 0.001$) only in the case of the first four rows of younger animals on each tray (Table IA); Figure 2A demonstrates that this is a result of a difference between the first three rows of tray A and those on trays B and C. Figure 2A illustrates also the similarity between trays for the older individuals and for the last five rows of younger animals.

The F-ratio for Factor 3 (row within tray) is highly significant for the last 5 rows of younger animals (Table IC), and significant at the $P < 0.02$ level for the rows of older individuals (Table IB). Figures 2A and B illustrate a consistent depression of final carapace length of greater than 15% in younger animals immediately downstream from the older individuals (rows 11–12), relative to the carapace lengths of those younger animals farther downsteam in rows 14–15 and also relative to those in rows 1–3 of the following tray. The corresponding depression in weight is about 40%. The second significant "row" effect is a result of a depression in older individuals' carapace lengths in the seventh, eighth, and ninth rows, relative to the fifth and tenth rows, in both systems (Fig. 2B) and at all three tray positions (Fig. 2A). Finally, when the first four rows of the last two trays only were subjected to ANOVA, the row variable just missed significance ($P < 0.064$). There is a suggestion in Figure 2A of depressed growth in younger animals of row 4 of tray C (just upstream from the block of older individuals).

We wished to determine the average rate at which the block of older animals' negative influence upon growth of the younger ones died away with distance. Accordingly, for each of the six trays, we regressed the natural logarithm of the difference between a reference value and the average carapace length in a row ($n = 8$) against its distance downstream from the older-animal block to obtain exponents for the corresponding geometric series model:

$$d_{\mathrm{i}} = d_0 e^{\;l\mathrm{i}}, \quad \mathrm{i} = 1, 2 \ldots \tag{7}$$

where d_{i} is the difference in mm between the carapace length for row i and the reference value, and l (in units of rows) is the reciprocal of the space constant of the series. We used a reference value of 0.1 mm larger than the largest row average in rows 11–15. The corresponding average value obtained for $\bar{l}$ was 0.690 ± 0.048. This value produces a "spatial half-life" for the downstream effect very close to one row.

Molt interval and increment differences

The 15% decrement in growth rate appears to be partitioned approximately equally between an increase in intermolt interval and a smaller molt increment. Carapaces, especially of the smaller molts, were often partially eaten before they were retrieved and measured, and many molts were missed, so that molt time information also was often lacking or uncertain. Hence, we subtracted the row

Table I

Analysis of variance, final carapace lengths.

Source	Degrees of freedom	Sum of squares	Mean square	F-ratio	P[1]
A. First four rows of younger animals (rows 1–4)					
Total	191	326.01			
Replicates	7	7.24	1.03	0.69	
System (1)	1	0.57	0.57	0.38	
Tray (2)	2	41.04	20.52	13.73	0.000
Interaction 12	2	3.35	1.92	1.29	>0.25
Row (3)	3	9.26	3.09	2.06	>0.10
Interaction 13	3	2.82	0.94	0.63	
Interaction 23	6	10.06	1.67	1.12	>0.35
Interaction 123	6	10.55	1.76	1.18	>0.30
Error	161	240.64	1.49		
B. Six older animal rows (rows 5–10)					
Total	287	599.73			
Replicates	7	16.29	2.33	1.14	>0.30
System (1)	1	4.72	4.73	2.32	>0.10
Tray (2)	2	7.68	3.84	1.89	>0.15
Interaction 12	2	4.15	2.08	1.02	>0.35
Row (3)	5	29.71	5.94	2.92	$0.02 > P > 0.01$
Interaction 13	5	4.20	0.84	0.41	
Interaction 23	10	10.40	1.04	0.51	
Interaction 123	10	23.80	2.37	1.17	>0.30
Error	245	498.76	2.04		
C. Last five rows of younger animals (rows 11–15)					
Total	239	652.32			
Replicates	7	9.47	1.35	0.63	
System (1)	1	1.86	1.86	0.88	
Tray (2)	2	0.70	0.35	0.16	
Interaction 12	2	2.35	1.17	0.55	
Row (3)	4	177.75	44.44	20.89	0.000
Interaction 13	4	1.26	0.32	0.15	
Interaction 23	8	8.95	1.12	0.52	
Interaction 123	8	16.36	2.05	0.96	
Error	203	433.61	2.14		

[1] Value only given for F > 1.0.

average for a particular molt number from that of the preceding one to estimate the interval or increment. For this reason standard errors are given only for molt time and carapace length in Table II, which summarizes the differences between the first and fifth rows of younger animals (*i.e.*, rows 11 and 15) following the older animal block in each tray.

The "nearest-neighbor effect" is felt immediately. Animals immediately downstream of the older individuals molt from fourth to fifth stage a day and a half later than those five rows downstream, and when their next molts are measured are found to be already 5 percent smaller. The quotient (b/a) combining the

TABLE II

Growth differences between first and fifth rows following the block of older animals, as reflected in intermolt interval and molt increment. Columns (a) and (b) express age and carapace length, respectively, of row 11 animals as percent of those in row 15. The last column estimates growth of row 11 animals as percent of those in row 15. Based upon final carapace length measurements this was 83.7%.

Molt to stage	Row	N	Age at molt (days)[1] $\bar{x} \pm$ s.e.	(a)	Intermolt interval (days) $(\bar{x}_i - \bar{x}_{i-1})$	N	Carapace length after molt (mm) $\bar{y} \pm$ s.e.	(b)	Molt increment (mm) $(\bar{y}_i - \bar{y}_{i-1})$	Estimated growth $\left(\frac{b}{a} \times 100\right)$
5	11	40	28.88 ± 0.39	105.7		22	5.355 ± 0.076	95.5	0.567	90.4
	15	34	27.33 ± 0.31			20	5.610 ± 0.089		0.810	
6	11	31	42.69 ± 0.44	107.81	13.81	21	6.256 ± 0.105	91.0	0.901	84.4
	15	41	39.60 ± 0.35		12.27	20	6.875 ± 0.124		1.265	
7	11	32	55.41 ± 0.60	107.25	12.70	20	7.590 ± 0.193	92.6	1.334	86.3
	15	40	51.66 ± 0.40		12.04	26	8.192 ± 0.122		1.317	
8	11	38	71.37 ± 0.50	107.63	15.96	23	9.083 ± 0.202	91.0	1.493	84.5
	15	42	66.31 ± 0.62		14.65	20	9.985 ± 0.238		1.793	
9	11	26	89.00 ± 1.01	107.0	17.63	12[2]	10.025 ± 0.336	85.5	0.942	79.9
	15	38	83.21 ± 0.77		16.90	27	11.719 ± 0.234		1.734	

[1] Taking Day 270/78 as age 0.

[2] Molt to stage 10 incomplete for this row by end of experiment.

effects of molt increment decrease (b) and molt interval increase (a) shows by the second molt in the system the full 15% decrease in growth seen in the final carapace-length figures. Upon exposure to 5th- to 6th-stage juveniles, the 4th-stage lobsterling apparently switches very soon to a slower rate of growth, reflected in both intermolt interval and molt increment.

DISCUSSION

Results of this study permit further elucidation of the density-dependent growth inhibition observed in earlier experiments with juvenile lobsters. The experimental design allowed separation of the effects upon growth of a) long-lived metabolites not removed by filtration, air-stripping, etc., which would thus build up along the table, and be much higher in concentration in System II with its lower makeup rate (see compartmental analyses above); b) long-lived metabolites removed by air-stripping or other means, which would build up along a table, but similarly in the two systems; and c) metabolites whose effects in the system were short-lived. The gradients in ammonia concentration may serve as a paradigm of a). Were such a metabolite effective in inhibiting growth in the concentrations attained, we would expect system differences, tray differences, and row differences to be manifest in the ANOVAs of Table I. Similarly, were metabolites which were removed in the sump, filters, or UV units effective in inhibiting growth in this experiment, we would expect only tray differences and row differences to appear in the ANOVAs. In fact, there were no differences at all between the systems, a result similar to that obtained by Cobb and Tamm (1974). The only tray differences were between the first three rows of trays A and the first three rows in trays B and C (Fig. 2A). (Surprisingly, growth in these first three rows in the gradient was *slower*. We have no ready explanations for this, although several possibilities exist. First, it is possible but not likely that supersaturation of gases in the seawater influent was not entirely removed by heating, air-stripping, and nucleation in the makeup

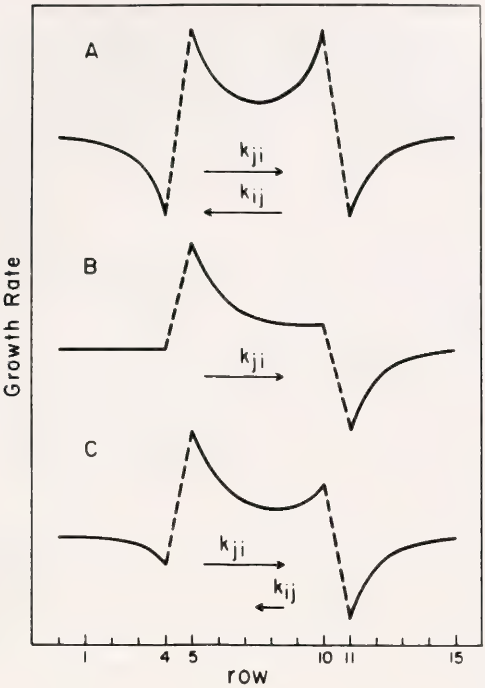


FIGURE 3. Compartment models corresponding to Figure 2. Growth rate (ordinate, arbitrary units) is plotted against compartment number in a catenary array (abscissa). Production of inhibitory principle is higher in compartments 5–10. In A, the turnover rate constants k_{ji} and k_{ij} (see compartmental analyses) are symmetrical; in B, the k_{ij}'s are negligible; in C the k_{ij}'s are small but not negligible.

sump (Fig. 1, M). Secondly, the UV treatment could have resulted, for example, in the production of short-lived ionized molecules with an adverse effect upon growth. Third, it is possible that "natural" sea water, either because of the presence of a substance removed by lobsters or through the absence of a "conditioning" substance produced by lobsters, is not as conducive to growth of lobsters as is lobster-conditioned water (see Cobb and Tamm, 1975a). But if the effect in our systems were attributable to the makeup water, it should be more pronounced in System I with the higher makeup fraction, which a separate two-factor factorial ANOVA on just the first four rows of trays A failed to show. Therefore, if a "conditioning" substance is responsible, it must be removed from the recirculation water before the latter re-enters tray A.)

Density-dependent growth inhibition appeared instead to be a short-lived effect of animals in the immediate neighborhood, *i.e.*, an effect which dies away with distance, and was obviously asymmetrical, *i.e.*, more pronounced downstream. Figure 3A indicates schematically what we would expect from a compartmental model (see compartmental analyses above) of a 15-compartment column with symmetrical upstream and downstream turnover constants k_{ij} and k_{ji} for the transmission of a short-lived inhibitory influence. Figure 3B shows the pattern anticipated were the upstream flow completely negligible, and Figure 3C the pattern with dominant downstream flow but non-negligible upstream influence. In the case of Figure 3A growth would be stunted symmetrically in the younger individuals on either side of the block of older animals, and also in the middle of that block. The reason for the latter effect is that the older animals at the edges of the block have only half the number of large neighbors as do the ones in the middle of the block and hence experience less inhibition. With model 3B only the older animals immediately downstream from the younger animals would be "released" from inhibition, and only the younger animals immediately downstream from the larger ones inhibited. If there were some slight upstream range of the inhibitory influence, the pattern of Figure 3C would result.

Comparison of Figure 3 to Figure 2 suggests the model of Figure 3C, and appears to eliminate certain explanations. First, the possibility of a direct behavioral cause, for example aggressive interactions, appears to be eliminated by the transmission of the effect to non-adjacent compartments. It is hard to imagine an effect based upon visual or bodily contact which could produce this result. Moreover, radiative influences (*e.g.*, sound) cannot account for the upstream-downstream asymmetry. We appear to be left with some form of chemical influence, carried differentially downstream, *but not accumulating*. Although in the dye tests no significant backflow of dye was observed, the possibility of small but appreciable upstream influence cannot be excluded.

There appear to be only two tenable hypotheses: first, the continuous production of a chemical substance or complex whose effect is short-lived, either because of loss to the outside or because of decay or inactivation within the system; and second, the pulsed production of a more or less long-lived inhibitory substance. Either process would be capable of producing an effect upon growth which died away with distance from the source. We will examine the two hypotheses in turn.

For a short-lived inhibitor with decay rate equal to the compartment turnover rate $k = 0.631$ per min, the steady-state concentrations in compartments downstream from the older-animal source may be calculated to be as shown in Figure 4B (see compartmental analyses above). For comparison we recall that the regressions for the downstream decay of the nearest-neighbor effect gave "spatial half-lives" of approximately one row for the downstream effect, as with these parameters. Thus we may say that if the growth inhibitory effect is due to a molecule or complex which decays or is otherwise lost from the system, the decay constant is on the order of 0.6/min or that the substance has a half-life of approximately 1 min. It appears from the oxygen results (see compartmental analyses above) that it is extremely unlikely that a substance could be lost through the air–water interface at such a rate, and surface absorption or bacterial inactivation at that rate seems similarily unlikely. If the molecule or complex is indeed lost from the system at this rate, it appears that it is unstable or easily inactivated by other factors in seawater.

If the inhibitor is produced in a pulse, as for example with intermittent urinary production (Cornell, 1979), the concentration pulse will "spread out" and become lower as it travels downstream, as in Figure 4A (using the compartment turnover rate $k = 0.631$/min; see compartmental analyses). With the pulsed-production model the question arises: To which aspect of the concentration is the downstream animal sensitive? If it integrates the concentration over time, then each downstream animal will experience the same quantitative effect upon growth. But if it is sensitive instead only to the maximum concentration experienced, or to the rate of increase in concentration in its compartment following the unit impulse upstream (in effect differentiating the curve of rising concentration), downstream effects upon growth rate similar to those observed could be obtained. Whatever parameter of the pulse is presumed effective, if its duration is much greater than a minute, or its onset is less abrupt than the unit impulse, downstream smoothing will be more pronounced and the successive compartments less differentiated from one another. Pulsed release of urine containing an inhibitor is a likely candidate. However, the hypothesis of a continuously produced short-lived molecular species or complex with a half-life on the order of a minute appears at this time to be equally tenable.

Without the blocks of older animals in the experiment, this rapidly-decaying effect upon growth would not have been demonstrated. However, that growth in the middle of these blocks is inhibited relative to the lead row of the block (Fig. 2) indicates that we are not just dealing with an effect of older upon younger animals, or of *H. americanus* upon hybrids. Even with cohorts of similar sized individuals such growth inhibition may be presumed to exist, and removal of it should result in economically significant gains in growth rate. But the best way to go about this will depend on experimental answers to the questions a) Is the inhibitory substance pulsed in its release, rapidly decaying, or both? and b) Is the substance self-inhibitory?

The fast-decaying growth inhibitory effect we have observed apparently differs in several respects from the delay-of-molt phenomenon described by Cobb and

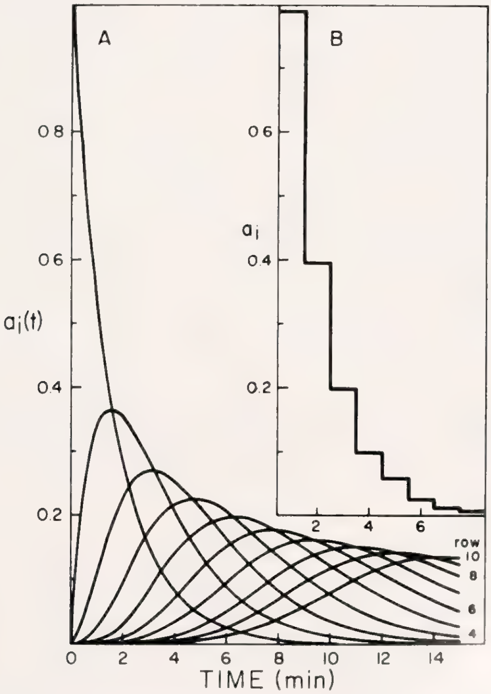


FIGURE 4. A. The course of concentration (ordinate) with time following a unit impulse of production (abscissa), in the successive compartments (rows) downstream from the production compartment (separate curves; the exponential curve represents the clearance of the production compartment). $A_0 = 1$ g per 1; $k = 0.631$ per min. See compartmental analyses.

B. Steady-state concentrations (ordinate) in successive rows (abscissa) downstream from a point of continuous production of an inhibitor decaying with a rate k_1 equal to the turnover rate to the next compartment k_2, both equal to 0.631/min. The rate of production has been set equal to 1 g per 1 min. See compartmental analyses.

Tamm (Cobb, 1968, 1970; Cobb and Tamm, 1974, 1975a–c). In their experiments the dominant individual of a pair held together clearly delays the molt of the subordinate individual, but no mention is made of an effect upon the growth increment of the subordinate. Furthermore, the molt-delay phenomenon is said to disappear in the absence of physical contact (Cobb and Tamm, 1975a). It seems instead to be an expression of dominance, possibly brought about in part by the greater activity of the subordinate animal (Cobb and Tamm, 1975c; the effect on growth which we have found could of course also be brought about by increased activity of the downstream animals). However, the molt-delay phenomenon is not clear in groups of three or more lobsters (Cobb and Tamm, 1975b). In further contrast to our results is their suggestion that chemical communication in the absence of visual or physical contact may facilitate molting rate and synchrony, although the evidence is apparently not convincing (Cobb and Tamm, 1975a). Our results point instead to a desynchronizing influence of the fast-decaying inhibitory effect. However, the possibility of chemical facilitation of growth (need for "lobster-conditioned" water) was discussed above as an explanation of the reduced growth rates in the first three rows of tray A (Fig. 2A).

The fast-decaying inhibitory effect together with dominance effects may account in full for the enhancement of growth rate variance in various crustaceans held communally (Cobb and Tamm, 1975b; Malecha, 1977; Aiken, 1977). In mass culture the dominants' behavior tends to isolate them from the more subordinate animals, which in turn often tend to crowd together in the corners, etc. (pers. obs.). Under such conditions a fast-decaying or pulsed inhibitor could exert maximum effects upon the subordinates. Whether such massing together occurs in nature is unknown. But it may under certain circumstances, *e.g.*, spatially concentrated food or shelter. Some of the so-called "space limitation" effects in crustacean culture experiments (Rauch *et al.*, 1974; Shleser, 1974; Sastry *et al.*, 1975; Sastry and French, 1977; Van Olst and Carlberg, 1978) may also be due to a fast-decaying inhibitory effect in compartment arrays which have not been designed to differentiate between the effects of limited space *per se* and the effects of nearest-neighbor inhibition (see also Cobb and Tamm, 1975c).

We wish to thank C. E. Falbo for mathematical suggestions, and L. W. Botsford, J. S. Cobb, and D. E. Wickham for their helpful comments on the manuscript.

SUMMARY

1. A density-dependent inhibitory effect upon the growth of juvenile lobsters was studied in two semi-recirculating seawater-table systems in which controlled metabolite gradients were established by varying rate of makeup flow. Each system contained eight columns of 45 compartments each. *Homarus americanus* fifth-stage juveniles were placed in the compartments in rows 5–10, 20–25, and 35–40, numbering from the incurrent end of each system, and fourth-stage *H. americanus* × *H. gammarus* F_1 hybrids were placed in all other compartments.

2. At the end of 93 days hybrids immediately downstream from the older animals were an average of 15% shorter than those five rows downstream. No effect of metabolite accumulation could be demonstrated.

3. Analysis by a compartmental model indicated that the growth-inhibitory effect was chemical and was due either to a rapidly decaying, continuously produced inhibitor with a half-life of about 1 min, or to an inhibitor pulsed in production or release.

4. Decreased growth in the middle of the blocks of older animals demonstrated that the effect was neither species- nor age-specfic. The effect is substantially different from other growth-inhibitory phenomena previously described in Crustacea.

LITERATURE CITED

AIKEN, D. E., 1977. Molting and growth in decapod crustaceans, with particular reference to the lobster *Homarus americanus.* Pages 41–73 in B. F. Phillips and J. S. Cobb, Eds., *Workshop on Lobster and Rock Lobster Ecology and Physiology.* CSIRO, Division of Fisheries and Oceanography, Melbourne.

ATKINS, G. L., 1969. *Multicompartment Models for Biological Systems.* Methuen and Co., Ltd., London, xiv + 153 pp.

COBB, J. S., 1968. Delay of moult by the larvae of *Homarus americanus. J. Fish. Res. Bd. Can.*, **25**: 2251–2253.

COBB, J. S., 1970. Effect of solitude on time between fourth and fifth larval molts in the American lobster (*Homarus americanus*). *J. Fish. Res. Bd. Can.*, **27**: 1653–1655.

COBB, J. S., AND G. R. TAMM, 1974. Social conditions increase intermolt period in juvenile lobsters, *Homarus americanus. J. Fish. Res. Bd. Can.*, **31**: 1941–1943.

COBB, J. S., AND G. R. TAMM, 1975a. Delay of molt in juvenile lobsters. Abstracts, p. 34 in J. S. Cobb, Ed., *Recent Advances in Lobster Aquaculture,* Sea Grant Technical Workshop, University of Rhode Island, Kingston, April 21, 1975.

COBB, J. S., AND G. R. TAMM, 1975b. Agonistic behavior of juvenile lobsters. Abstracts, p. 36 in J. S. Cobb, Ed., *Recent Advances in Lobster Aquaculture,* Sea Grant Technical Workshop, University of Rhode Island, Kingston, April 21, 1975.

COBB, J. S., AND G. R. TAMM, 1975c. Dominance status and molt order in lobsters (*Homarus americanus*). *Mar. Behav. Physiol.,* **3**: 119–124.

CORNELL, J. C., 1979. Salt and water balance in two marine spider crabs, *Libinia emarginata* and *Pugettia producta.* I. Urine production and magnesium regulation. *Biol. Bull.*, **157**: 221–233.

DAGGETT, R., AND D. ARONSTEIN, 1979. *Second Annual Seawater Quality Report,* University of California, Bodega Marine Laboratory, Bodega Bay, California, 12 pp.

DELISTRARY, D. A., J. M. CARLBERG, J. C. VAN OLST, AND R. F. FORD, 1977. Ammonia toxicity in cultured larvae of the American lobster (*Homarus americanus*). *Proc. World Mariculture Soc.*, **8**: 647–672.

HAND, C., T. ALBANY, L. BOTSFORD, D. CONKLIN, R. DAGGETT, B. FISHER, M. HARTMAN, D. HEDGECOCK, K. NELSON, AND E. NILSON, 1977. *Development of Aquaculture Systems,* University of California, Sea Grant Publication No. 58, La Jolla, California. 97 pp.

HEDGECOCK, D., K. NELSON, AND R. A. SHLESER, 1976. Growth differences among families of the lobster, *Homarus americanus. Proc. World Mariculture Soc.*, **7**: 347–361:

HEDGECOCK, D., AND K. NELSON, 1978. Components of growth rate variation among laboratory cultured lobsters (*Homarus*). *Proc. World Mariculture Soc.,* **9**: 125–137.

LIAO, P. B., AND R. D. MAYO, 1972. Salmonid hatchery water reuse systems. *Aquaculture*, **1**: 317–335.

LOGAN, D. T., AND C. E. EPIFANIO, 1978. A laboratory energy balance for the larvae and juveniles of the American lobster *Homarus americanus. Mar. Biol.*, **47**: 381–389.

MALECHA, S. R., 1977. Genetics and selective breeding of *M. rosenbergii.* Pages 328–351 in J. A. Hanson and H. L. Goodwin, Eds., *Shrimp and Prawn Farming in the Western Hemisphere,* Dowden, Hutchinson and Ross, Inc., Stroudsburg, Pennsylvania.

MOFFETT, W. L., AND W. S. FISHER, 1978. Ammonia production rates of *Artemia salina* under various culture conditions. *J. Fish. Res. Bd. Can.*, **35**: 1643–1648.

RAUCH, H. E., L. W. BOTSFORD, AND R. A. SHLESER, 1974. Economic optimization of an aquaculture facility. *IEEE Trans. on Automatic Control,* **20**: 310–319.

Sastry, A. N., D. Wilcox, and J. Laczac, 1975. Effects of space on growth and survival of lobsters, *Homarus americanus*. Abstracts, page 39 in J. S. Cobb, Ed., *Recent Advances in Lobster Aquaculture*, Sea Grant Technical Workshop, University of Rhode Island, Kingston, April 21, 1975.

Sastry, A. N., and D. P. French, 1977. Growth of American lobster, *Homarus americanus* Milne-Edwards, under controlled conditions. P. 11 in B. F. Phillips and J. S. Cobb, Eds., *Workshop on Lobster and Rock Lobster Ecology and Physiology*, CSIRO Division of Fisheries and Oceanography, Melbourne.

Shleser, R. A., 1974. Studies of the effects of feeding frequency and space on the growth of the American lobster, *Homarus americanus*. *Proc. World Mariculture Soc.*, **5**: 149–155.

Standard Methods for the Examination of Water and Wastewater, 1976 (14th Edition). American Public Health Association, Washington, D. C., 1193 pp.

Van Olst, J. C., and J. M. Carlberg, 1978. The effects of container size and transparency on growth and survival of lobsters cultured individually. *Proc. World Mariculture Soc.* **9**: 469–479.

Reference: *Biol. Bull.*, **159**: 177–192. (August, 1980)

SEASONAL ABUNDANCE AND DISTRIBUTION OF *CRANGON FRANCISCORUM* AND *PALAEMON MACRODACTYLUS* (DECAPODA, CARIDEA) IN THE SAN FRANCISCO BAY-DELTA[1]

CLIFFORD A. SIEGFRIED

Biological Survey, New York State Education Department,
Cultural Education Center, Albany, New York 12230

Two decapod shrimp, *Crangon franciscorum* and *Palaemon macrodactylus*, dominate the epifaunal community of the western Sacramento-San Joaquin Delta. The Franciscan bay shrimp, *C. franciscorum*, once supported an extensive commercial fishery in San Francisco Bay (Bonnot, 1932) and today supports a more restricted bait fishery. It is a common inhabitant of the continental shelf and estuaries from southeastern Alaska to California (Schmitt, 1921). Israel (1936) discussed the life history of *C. franciscorum* in the San Francisco Bay-Delta region and Ganssle (1966) provided additional qualitative information on its distribution and abundance in Suisun Bay. Recent studies have investigated aspects of the physiology and tolerance of *C. franciscorum* to various environmental factors (Khorram and Knight, 1977; Sharp *et al.*, 1978; Nelson *et al.*, 1979) but little information is available on the biology of *C. franciscorum* in the Bay-Delta.

The oriental shrimp, *Palaemon macrodactylus*, is thought to have been introduced into the San Francisco Bay Estuary in the early 1950s from water-ballast tanks of ships returning from Korea (Newman, 1963). Its presence in San Francisco Bay is thought to represent its northernmost distribution on the west coast of North America. Little is presently known about the biology of the oriental shrimp in the Bay-Delta system.

C. franciscorum and *P. macrodactylus* are important components of the estuarine food web, especially as food for the game fish of the estuary (Ganssle, 1966). These shrimp are also important predators of the opossum shrimp, *Neomysis mercedis*, (Siegfried *et al.*, 1978; Sitts, 1978) and may be important in nutrient cycling in the estuary (Nelson *et al.*, 1979). Information on potential interactions between the native specimens of *C. franciscorum* and the introduced specimens of *P. macrodactylus* is lacking.

MATERIALS AND METHODS

Decapod shrimp were collected from 5 to 12 stations on the Sacramento River, from Chipps Island upstream to Rio Vista, from January, 1976, through April, 1977; and from 32 stations in Suisun Bay and the Sacramento and San Joaquin Rivers from April, 1977, through October, 1978 (Fig. 1). In 1976 a few shallow, near shore stations (< 4 m deep) were sampled but in 1977–1978 all stations were located near midchannel and were 6–14 m deep. During the initial phase of the study (January, 1976–April, 1977) decapod shrimp were collected monthly by replicate tows with three #8 nets (mesh = 200 μm) towed simultaneously at each

---

[1] New York State Museum Journal Series No. 285.

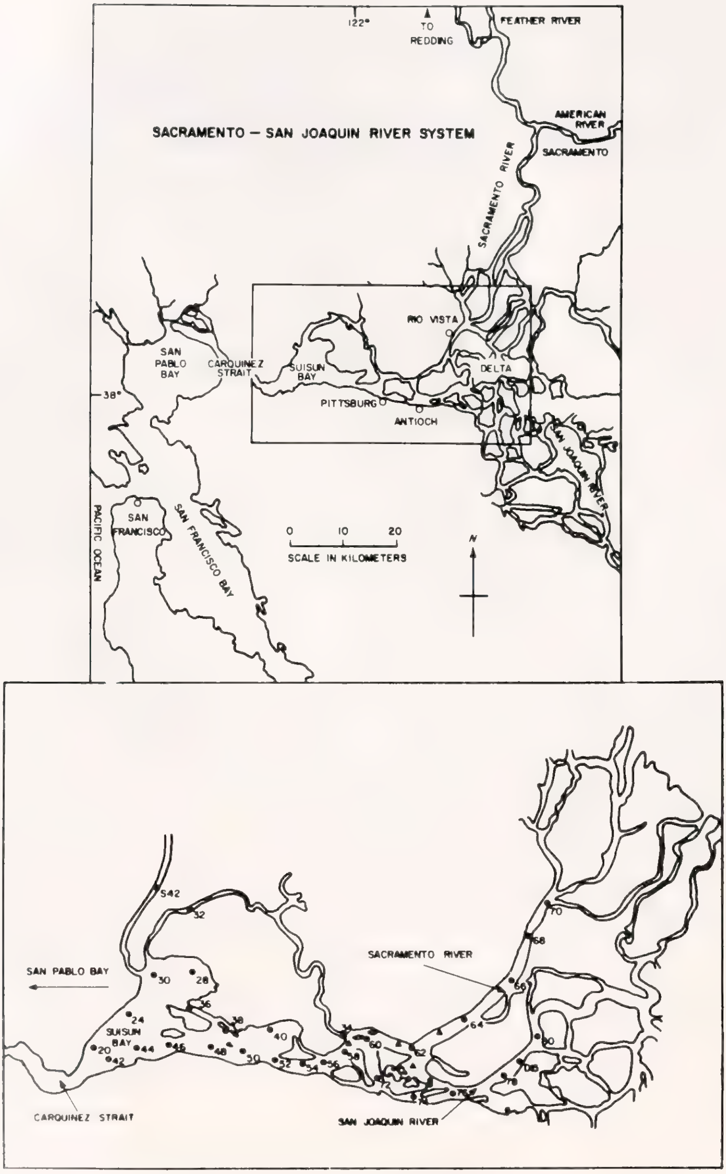

122°
TO REDDING
FEATHER RIVER
SACRAMENTO – SAN JOAQUIN RIVER SYSTEM
AMERICAN RIVER
SACRAMENTO
SACRAMENTO RIVER
RIO VISTA
DELTA
SAN PABLO BAY
CARQUINEZ STRAIT
SUISUN BAY
38°
PITTSBURG
ANTIOCH
SAN JOAQUIN RIVER
SAN FRANCISCO
SAN FRANCISCO BAY
PACIFIC OCEAN
0 10 20
SCALE IN KILOMETERS
N
S42
32
SAN PABLO BAY
30
28
36
24
SUISUN BAY
20
42
44
46
38
40
48
50
52
54
56
34
58
60
62
64
66
68
70
SACRAMENTO RIVER
80
76
CARQUINEZ STRAIT
SAN JOAQUIN RIVER

Figure 1. Location of study area and collection sites in Suisun Bay and the Sacramento-San Joaquin River Delta. Triangles indicate sites sampled only in 1976.

station: one just below the surface, one near mid-depth, and the third secured to a sled which maintained it just above the substrate. The nets consisted of a 1.3-m section of cylindrical netting, 50 cm in diameter, preceding a 2-m cone-shaped section which terminated in a removable plankton bucket. Each net was equipped with a current meter which was used to determine the volume sampled. From April, 1977, through October, 1978, single step-wise tows were made at biweekly intervals at the 32 stations indicated in Fig. 1. The nets used during this phase of the study were of 505-μm mesh netting, with 29-cm-diameter mouths and were 1.48-m long. These tows were made during a 3–5 consecutive days between ½ hr before and 1 hr after high neap tide by the California Department of Fish and Game, Bay–Delta Fishery Project, as part of their *N. mercedis* monitoring program. Conductivity and water-temperature data were collected at each sample site. All shrimp collected were placed in 10% buffered formalin and later transferred to 70% isopropyl alcohol.

All shrimp collected were measured from the anterior edge of the carapace (excluding the rostrum) to the posterior tip of the telson. The sex of all specimens collected in 1977 and 1978 was determined by microscopic examination of the endopodite of the second pleopod. On developing and mature males this endopodite bears an appendix masculina, whereas for females the structure is similar to those of the third pleopod (Newman, 1963; Smith and Carlton, 1975). An additional distinguishing characteristic used for *C. franciscorum* sex determination was the structure of the endopodite of the first pleopod, which is short and curved inward for males and long and straight for females. Preservation made it difficult to locate the gonopore of each shrimp so this character was not used for sex determination.

For brood-size determinations the eggs of 17 ovigerous specimens of *C. franciscorum* and 66 specimens of *P. macrodactylus* were stripped and enumerated. Only females with ova in the early developmental stages were used for determination of brood sizes.

Length-weight relationships were determined for 238 specimens of *C. franciscorum* and 183 specimens of *P. macrodactylus*. Each shrimp was measured and then dried at 60°C for 48 hr, cooled, and then weighed to the nearest 0.01 mg for shrimp > 2 mg and to the nearest 0.001 mg for shrimp < 2 mg.

RESULTS

The size frequency distribution of specimens of *C. franciscorum* collected in the study area from November, 1977, through October, 1978, is presented in Figure 2. Specimens of *C. franciscorum* were not caught until they were about 10 mm long. The largest influx of 10–20-mm-long *C. franciscorum* specimens occurred in May. Sexual dimorphism is typical for crangonids (Lloyd and Yonge, 1947; Meredith, 1952; Allen, 1960; Price, 1962; Durkin and Lipovsky, 1977) and was evident for specimens of *C. franciscorum* from the Bay-Delta. Almost all specimens of *C. franciscorum* collected from the delta that exceeded 45 mm in length were female. The largest female collected from the delta was 72 mm long while the largest male was 52 mm long. Collections of *C. franciscorum* made in San Pablo Bay in May–June and September, 1977, indicate a significantly different population structure (Fig. 3). Samples from the delta in May–June, 1977, indicated a population made up of about 50% juvenile shrimp, median size = 34 mm and maximum size = 50 mm. Samples from San Pablo Bay indicate a population primarily of mature shrimp, median size = 50 mm and maximum size = 72 mm. In September the median of *C. franciscorum* collected in the delta was 38 mm (range 27–52 mm) while in San Pablo Bay the medium size was 48 mm (29–64 mm).

No estimates of growth were made from the size frequency histograms. Immigration, emigration, and temperature–salinity differences all combine to obscure growth patterns of *C. franciscorum* in the Delta. *C. franciscorum* eggs generally hatch in the spring. Young develop into juveniles by summer and reach maturity by winter.

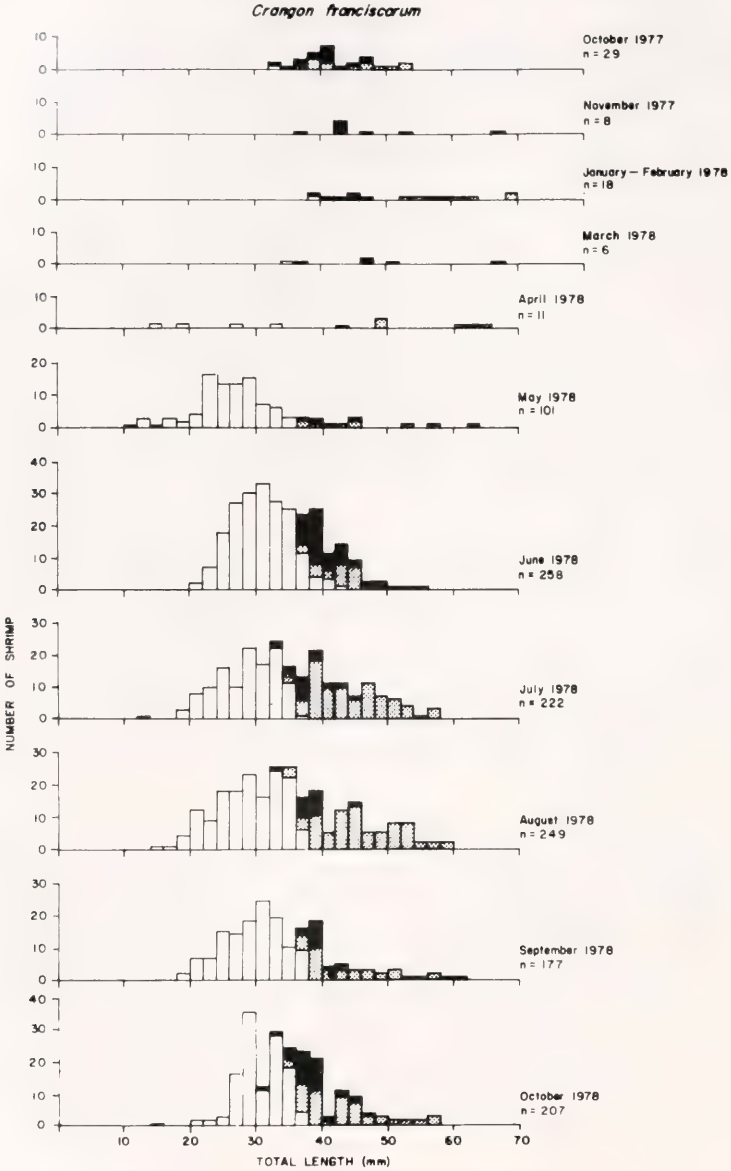


FIGURE 2. Size frequency distribution of specimens of *Crangon franciscorum* collected in the Suisun Bay, Sacramento-San Joaquin River study area, November, 1977–October, 1978. Dark bars indicate number of males, hatched bars indicate number of females, and open bars indicate number of juveniles or undetermined individuals measured.

The size frequency distribution of specimens of *P. macrodactylus* collected in the study area is presented in Figure 4. Summer is the main recruitment period for *P. macrodactylus* in the delta. Ovigerous females were collected from May through August, with a few collected in April and September. *P. macrodactylus* larvae are planktonic and are very abundant in the plankton of the delta during summer. Larval specimens of *P. macrodactylus* are photopositive (Little, 1969) but become more photonegative as they develop (Siegfried *et al.,* 1978).

Sexual dimorphism is also apparent in *P. macrodactylus*. Almost all individuals longer than 40 mm were female. Females reached *ca.* 55 mm maximum length and males *ca.* 44 mm. This is near previously reported sizes (Newman, 1963). Size at sexual maturity was not determined but secondary sexual characteristics are apparent when shrimp attain about 20 mm in length. Ovigerous females as small as 23 mm long were collected, so males would presumably mature at this or smaller sizes.

Length–weight relationships for juvenile, male and female specimens of *C. franciscorum* are presented in Figure 5. The regression equations describing these relationships are given below:

Juveniles (n = 100) : $\log W = -5.41 + 2.58 \log L$, r = 0.88
Males (n = 74) : $\log W = -6.12 + 3.27 \log L$, r = 0.92
Non-ovigerous females (n = 57) : $\log W = -6.62 + 3.57 \log L$, r = 0.96

(W = dry weight in grams and L = total length in mm). Analysis of covariance (Steel and Torrie, 1960) revealed significant differences in slopes between the length–weight regression of juveniles and mature shrimp. The difference is attributable, at least in part, to gonadal development.

Length–weight relationships for *P. macrodactylus* are presented in Figure 6. Regression equations describing these relationships are given below:

Juveniles (n = 118) : $\log W = -5.44 + 2.53 \log L$, r = 0.95
Males (n = 45) : $\log W = -5.49 + 2.95 \log L$, r = 0.97
Non-ovigerous females (n = 19) : $\log W = -6.10 + 3.40 \log L$, r = 0.98

The slopes of the above regressions are all significantly different from one another ($\alpha = 0.05$). *P. macrodactylus* is more robust than *C. franciscorum*, in many cases weighing 50% more than a *C. franciscorum* of similar length.

Ovigerous specimens of *C. franciscorum* were collected in San Pablo and San Francisco Bays in the spring and fall of 1977. Most female specimens of *C. franciscorum* collected in San Francisco Bay in May were ovigerous while in

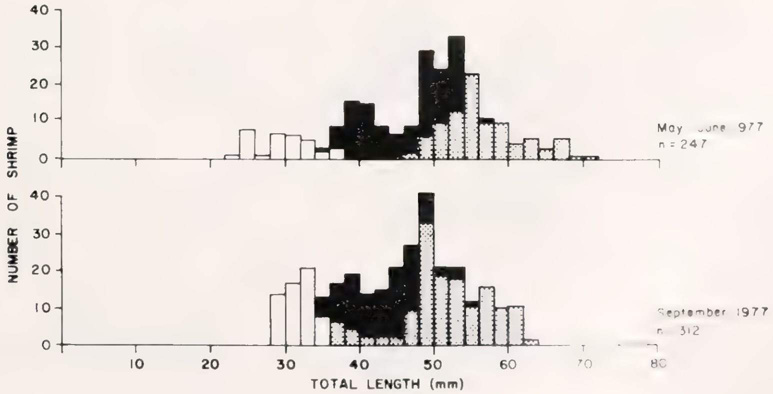


FIGURE 3. Size frequency distribution of specimens of *Crangon franciscorum* collected in San Pablo and San Francisco Bays, May–June and September, 1977. Dark bars indicate number of males, hatched bars indicate number of females and open bars indicate number of juveniles or undetermined individuals measured.

September–October, most were non-ovigerous. Ovigerous females in our samples ranged from 48 to 67 mm long.

Ovigerous specimens of *P. macrodactylus* were collected in the study area from April (1977) and May (1976, 1978) through August each year. More than

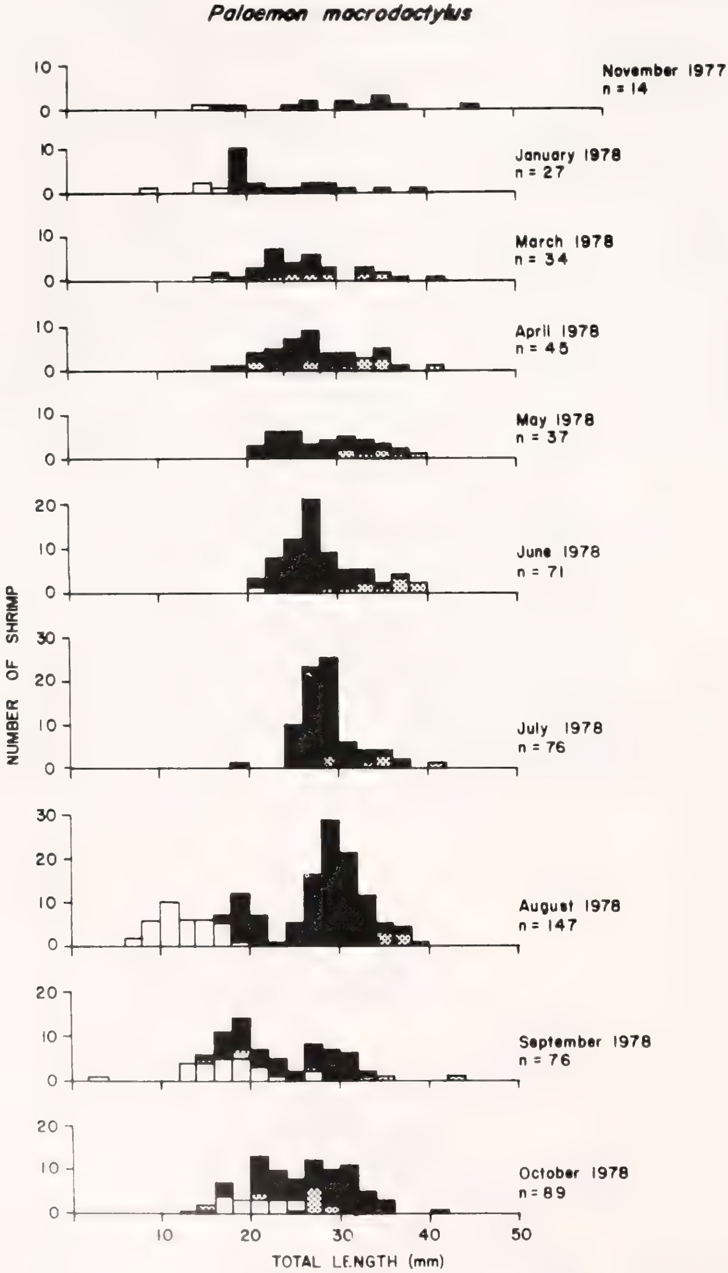


FIGURE 4. Size frequency distribution of specimens of *Palaemon macrodactylus* collected in Suisun Bay, Sacramento-San Joaquin River study area, November, 1977. Dark bars indicate number of males, hatched bars indicate number of females, and open bars indicate number of juveniles or undetermined individuals measured.

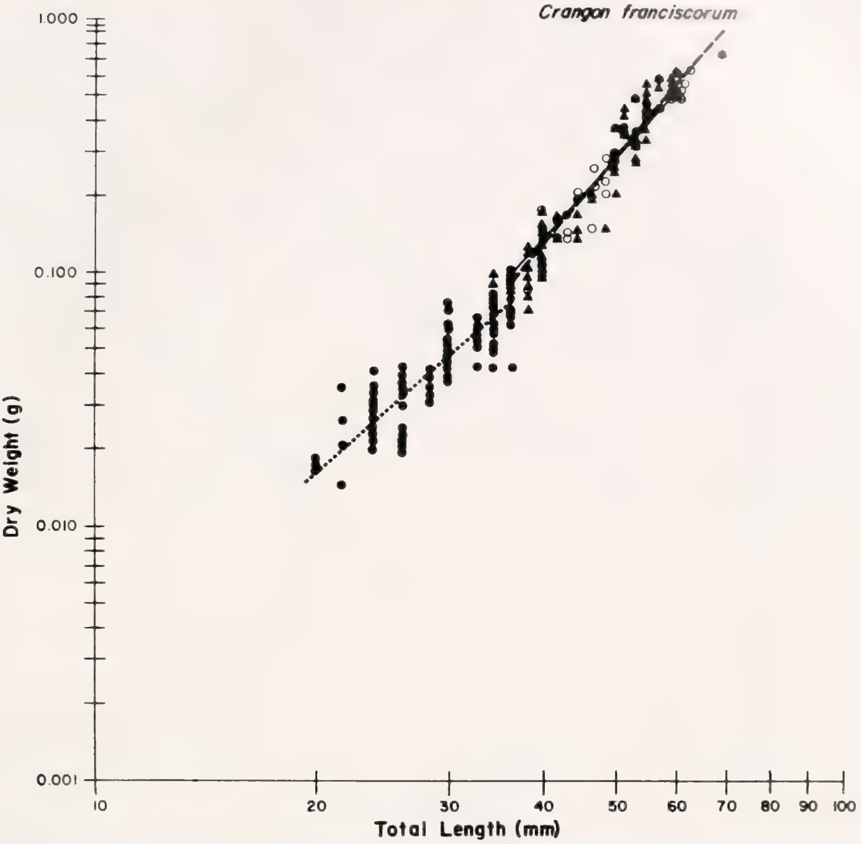


FIGURE 5. Relationship between total length (mm) and dry weight (g) of juvenile (solid circles), male (open circles), female (triangle) specimens of *Crangon franciscorum* collected from the study area, January, 1976–October, 1978.

50% of the mature females collected during these periods were ovigerous. Ovigerous females ranged in size from 23 to 48 mm long. Female specimens of *P. macrodactylus* appear capable of producing more than one brood; ovigerous females often carried a second brood in the ovaries while the first brood had not yet been released.

The brood sizes of 17 specimens of *C. franciscorum*, ranging in size from 48 to 67 mm long, and 66 specimens of *P. macrodactylus* ranging in size from 23 to 48 mm long, are presented in Figure 7. The relationships between brood size and body length are given by: *C. franciscorum:* $\log N = -3.66 + 4.09 \log L$, $r = 0.90$; *P. macrodactylus:* $\log N = -1.66 + 2.96 \log L$, $r = 0.81$. The size ranges of ovigerous females of the two species have little overlap, but in the area of overlap, *P. macrodactylus* has a greater mean brood size than *C. franciscorum*.

The seasonal abundance of *C. franciscorum* and *P. macrodactylus* in various parts of the study area is shown in Figures 8 and 9. There are obvious differences between 1977 and 1978 in the location of the peak population densities. In 1977, a very dry year, salinity incursion was more extensive than in 1978, and the peak densities of both *C. franciscorum* and *P. macrodactylus* were located in the river channels. In 1978 the bulk of both populations was centered in Suisun Bay.

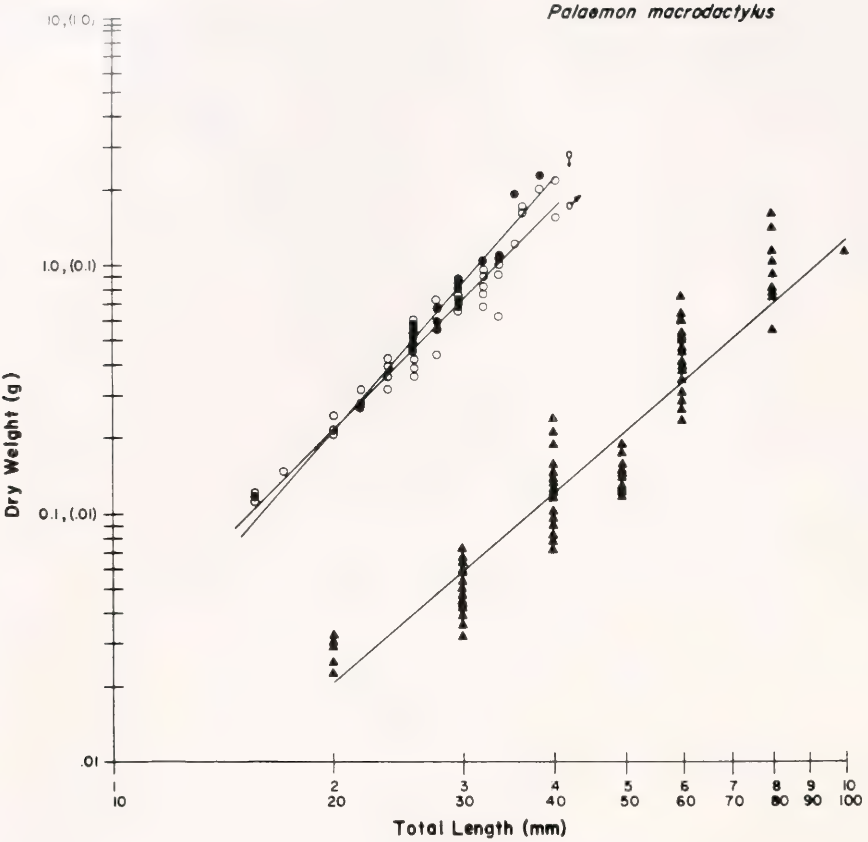


FIGURE 6. Relationships between total length (mm) and dry weight (g) of juvenile (triangle), male (open circle), female (solid circle) specimens of *Palaemon macrodactylus* collected from study area, January, 1976–October, 1978. Inner scale = juveniles, outer scale = male and female shrimp.

A significant difference between *C. franciscorum* and *P. macrodactylus* is evident in their population densities in the San Joaquin River. *P. macrodactylus* was abundant in the San Joaquin River during the summer (particularly in 1977) while *C. franciscorum* was virtually absent. The San Joaquin River receives more industrial and agricultural effluents relative to its discharge than the Sacramento River. This may create water quality differences between the two rivers that limit *C. franciscorum* distribution.

Both *C. franciscorum* and *P. macrodactylus* appeared to be limited upstream by low salinities. Few individuals of either species were collected from waters <1‰ salinity (Fig. 10). In both 1977 and 1978, the maximum concentration of *C. franciscorum* in the delta was generally in the salinity range 1–7‰ (Fig. 10). *Palaemon* appears to be more tolerant of low salinities combined with low temperatures than is *C. franciscorum*. In the spring and fall–winter it is not unusual to collect *P. macrodactylus* in fresh or nearly fresh water. *C. franciscorum* is generally absent from the delta channels at those times but is often abundant in Suisun Slough and adjacent sloughs from January through May where salinities are near 2‰.

Discussion

The maximum size of *C. franciscorum* collected in this study is in good agreement with maximum sizes reported from Yaquina Bay, Oregon (Krygier and Horton, 1975). However, collections of *C. franciscorum* off the mouth of the Columbia River indicate a population with mean size > 80 mm and maximum sizes > 110 mm (Durkin and Lipovsky, 1977). Reduction of maximum size of individuals inhabiting estuaries as compared to individuals of the same species inhabiting the sea has been reported for *Crangon crangon* (Maucher, 1961). Remane and Schlieper (1971) suggest that reduction in size of marine animals, although generally slight in higher Crustacea living in brackish water, is comparable to Bergmann's law—that is, size is related to features of the physical environment. The reduction may be attributable to physiological effects of salinity, reduced food availability, or a combination of these and other factors. Studies of osmotic regulation indicate that smaller specimens of *C. franciscorum* are capable of better hyper-regulation than larger individuals and that larger ones may tend to hypo-regulate better than smaller individuals (Shaner, 1978). Thus, the migration of larger individuals from low salinity waters to high salinities would be energetically advantageous.

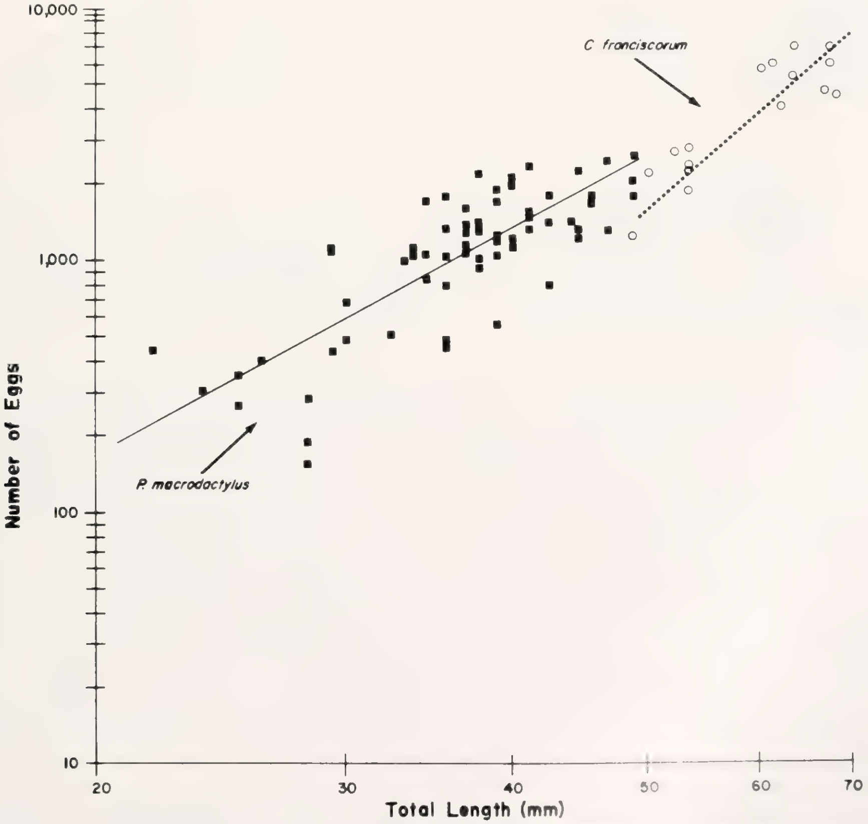


Figure 7. Relationship between total length (mm) and number of eggs carried by ovigerous specimens of *Crangon franciscorum* and *Palaemon macrodactylus*. All ovigerous specimens of *Crangon franciscorum* were collected in San Pablo Bay. All ovigerous specimens of *Palaemon macrodactylus* were collected in the study area.

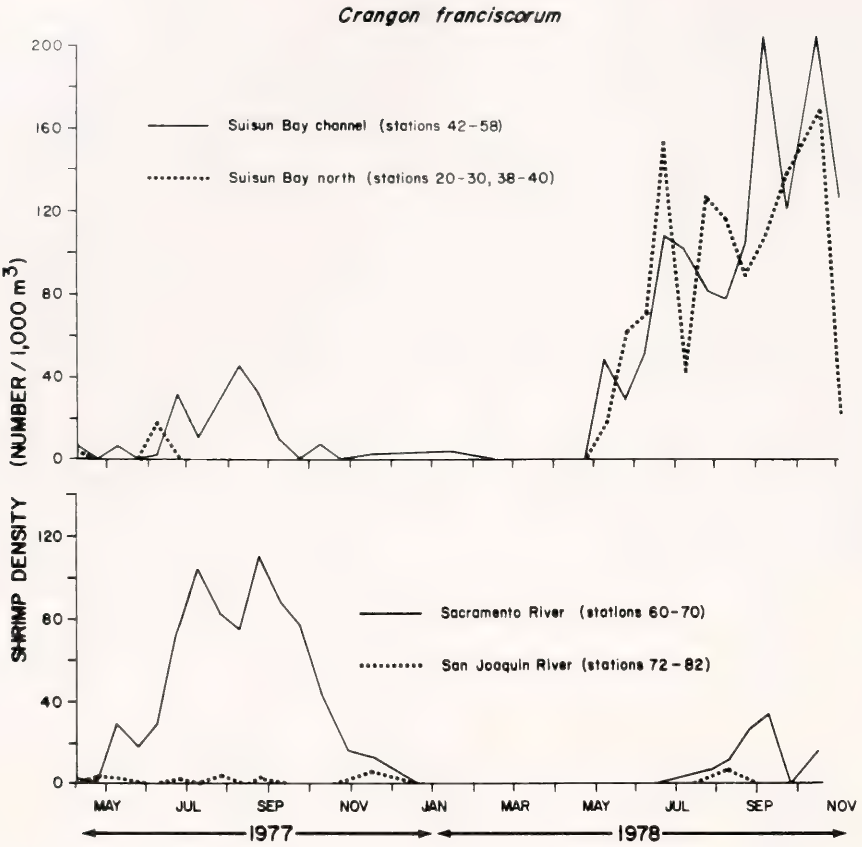


FIGURE 8. Abundance of *Crangon franciscorum* in various portions of the study area, April, 1977–October, 1978.

Although *C. franciscorum* inhabits brackish water for much of its life cycle in the delta, it requires high salinities for reproduction. No ovigerous female specimens of *C. franciscorum* were collected in the delta at any time during the study. Ovigerous females are found year-round in San Pablo and San Francisco Bays but the peak reproductive period appears to be from December through June (Isreal, 1936). Energetic demands of osmoregulation at low salinities may preclude egg development and thus reproduction in the delta. Broekema (1941) has shown that low salinities, characteristic of the delta, retard egg development in crangonids. Krygier and Horton (1975) report collecting only 1 of 120 ovigerous specimens of *C. franciscorum* at salinities below 15‰. Salinity is important not only in relation to egg development but also in larval survival. Preliminary investigation suggest that survival of larval specimens of *C. franciscorum* declines at salinities below 12‰ (Shaner, unpublished).

The life history pattern of *C. franciscorum* in San Francisco Bay appears not only to minimize the energetic demands of osmoregulation but also to minimize interspecific competition with another crangonid that is abundant in San Francisco Bay, *C. nigracauda*. These species coexist over much of their range (Krygier and Horton, 1975), partitioning their habitat temporally and spatially. In San Francisco Bay *C. franciscorum* spawns earlier in the year than *C. nigracauda* and moves

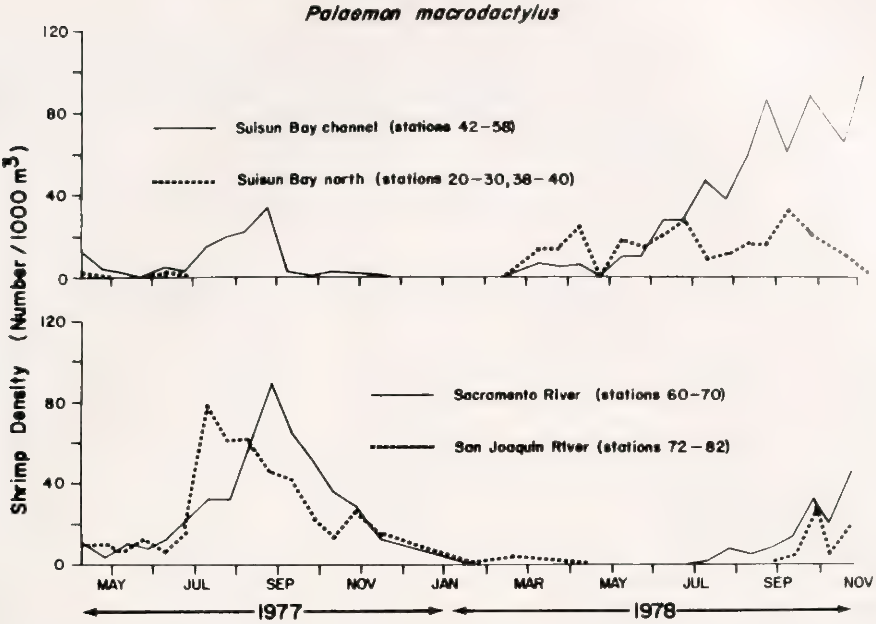


FIGURE 9. Abundance of *Palaemon macrodactylus* in various portions of the study area, April, 1977–October, 1978.

into the brackish waters of the delta in the summer. *C. nigracauda* is restricted to the higher salinity waters of the bay and ocean where it spawns during the summer months (Isreal, 1936; Siegfried, unpublished). *C. nigracauda* was collected only rarely, and then only at the highest salinities, from the study area. *C. franciscorum* is also somewhat larger than *C. nigracauda,* suggesting the potential for trophic resource partitioning based on size differences. A third crangonid, *C. nigramaculata,* also occurs in San Francisco Bay but little is known of its biology in the system.

The life history of *P. macrodactylus* differs in several important respects from that of *C. franciscorum.* Recruitment of young specimens of *P. macrodactylus* is separated both temporally and geographically from that of specimens of *C. franciscorum.* These differences reduce niche overlap between these shrimp and may provide an alternate prey for the *C. franciscorum* population. Larval and post-larval specimens of *P. macrodactylus* are preyed upon by specimens of *C. franciscorum* and may provide a "buffer" during periods of low prey availability (Siegfried, in preparation). Temporal separation of recruitment accentuates size differences between populations of *C. franciscorum* and *P. macrodactylus* and enhances trophic resource partitioning between these shrimp (Siegfried, in preparation). Differences in size and spawning season may be important factors ultimately determining the potential for coexistence of these two shrimp in the San Francisco Bay System.

Reproduction by specimens of *P. macrodactylus* is influenced by temperature, salinity, and photoperiod. Observations of *P. macrodactylus* at higher salinities in the San Francisco Bay system indicate that ovigerous females are found for a longer period than in the brackish water of the delta, *i.e.,* mid-April to late October (Little, 1969). Photoperiod appears to be an important parameter controlling spawning in *P. macrodactylus.* Spawning in the laboratory did not begin until

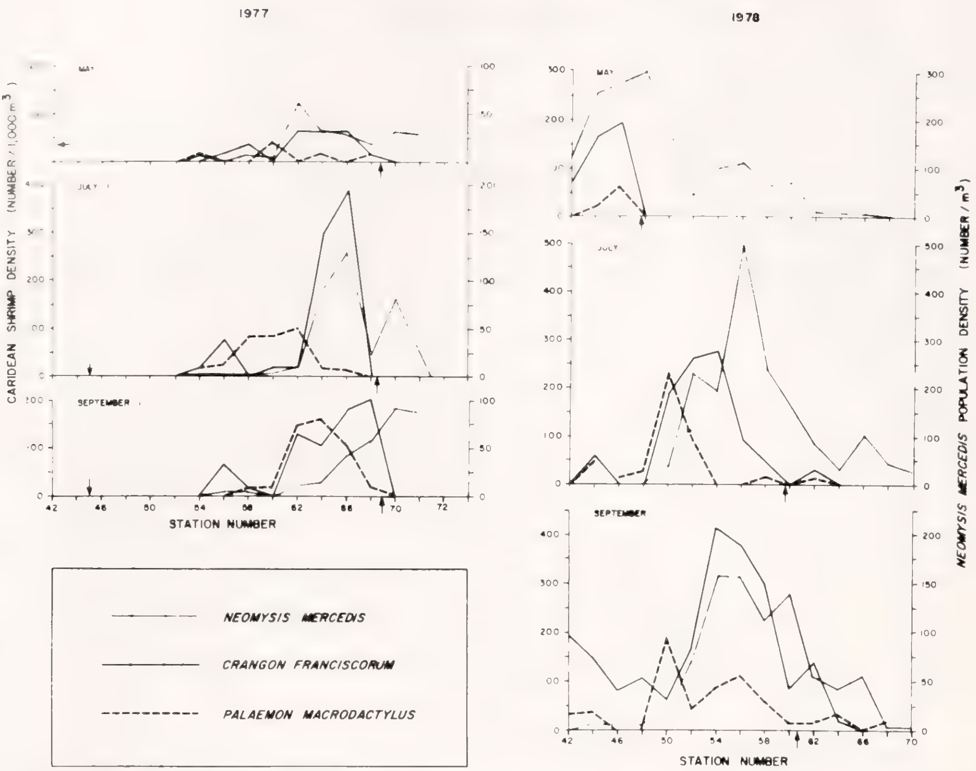


FIGURE 10. Abundance of *Crangon franciscorum*, *Palaemon macrodactylus*, and *Neomysis mercedis* at stations 42–70, on selected dates, May, 1977–September, 1978. Arrow pointing upward marks 1‰ salinity, arrow pointing downward marks 18‰ salinity.

the photoperiod was above 12 hr light : 12 hr dark, *i.e.* March, and occurred repeatedly at 20°C under 16 hr photoperiod (Barclay, 1978).

Laboratory bioassay indicates optimum salinity for adult *C. franciscorum* to be around 18–20‰ (Khoram and Knight, 1977). In 1977 this salinity range was present in lower Suisan Bay (stations 42–46) yet no specimens of *C. franciscorum* were collected from that area in 1977 (Fig. 9). During 1978 that salinity range was located downstream of the study area throughout the year. More recent salinity tolerance investigations indicate juvenile *C. franciscorum* to be more tolerant of low salinity, with 100% survival at 2‰ (Shaner, unpublished). Although low salinity limits the upstream distribution of specimens of *C. franciscorum*, other factors are important in determining their downstream distribution.

The distribution of *C. franciscorum* in the delta is influenced by the availability of its principal prey species, *N. mercedis*. Analysis of gastric mill contents of *C. franciscorum* indicates that not only is *C. franciscorum* density much higher in locations were *N. mercedis* density is high (Fig. 9), but those individuals of *C. franciscorum* in areas of high prey density take more prey than those from low prey density areas (Siegfried, in preparation). The dearth of other populations of suitable prey species in the delta region may be an important factor linking the distribution of the crangonid population to that of *N. mercedis*.

TABLE I.

Calculated indices of spatial overlap (L), and interspecific crowding between C. franciscorum *and* P. macrodactylus *(Z) in the San Francisco Bay Delta, April 1977–October 1978. See text for explanation. Roman numerals = different collection series.*

Sample series	L_{xy}		$Z_{c(p)}$		$Z_{p(c)}$		Mean No. specimens of *Crangon*/ Station		Mean No. specimens of *Palaemon* Station	
	1977	1978	1977	1978	1977	1978	1977	1978	1977	1978
January		1.84		1.61		1.50		0.81		0.88
February		0		0		0		0.12		0.35
March I		0.06		1.00		0.06		0.03		0.49
March II		0		0		0.20		0.14		0.46
April I	0	0	0	6.00	.0	0.36	0.14	0.05	0.66	0.89
April II	1.42	3.47	0.33	1.22	0.11	0.85	0.13	0.24	0.23	0.35
May I	1.31	3.99	0.59	1.41	0.76	4.77	0.44	1.19	0.33	0.35
May II	0	2.12	0	2.67	0	7.27	0.17	1.62	0.20	0.59
June I	1.17	3.24	0.30	3.02	0.60	9.06	0.51	2.59	0.26	0.86
June II	1.94	2.36	1.27	3.02	2.48	12.00	1.15	4.73	0.59	1.19
July I	0.89	3.42	1.18	3.79	0.98	10.26	1.15	3.07	1.38	1.13
July II	2.59	1.57	3.11	2.33	4.30	6.77	1.67	4.27	1.21	1.47
August I	3.66	0.64	5.60	1.62	4.85	2.56	1.33	4.03	1.54	2.57
August II	3.51	1.02	7.15	3.30	5.89	5.49	1.67	4.27	2.03	2.57
September I	4.76	2.42	4.89	4.04	4.53	8.03	0.95	6.10	1.03	3.07
September II	6.66	1.00	5.24	4.44	5.77	4.82	0.85	4.67	0.77	4.30
October I	5.00	1.62	2.85	4.01	2.59	9.12	0.51	7.20	0.56	3.17
October II	2.09	1.16	2.00	3.57	0.43	3.57	0.21	3.07	0.95	3.07
November	2.53		0.78		0.58		0.23		0.31	

The diet of *P. macrodactylus* in the delta consists almost entirely of *N. mercedis* (Siegfried, in preparation). However, the numerical and functional response to prey density is not as strong as in *C. franciscorum*. *P. macrodactylus* appears, like *C. franciscorum*, to be limited downstream by the lack of prey and upstream by low salinities. Although the peak *P. macrodactylus* densities often coincide with high *N. mercedis* densities (Fig. 9), the maximum *P. macrodactylus* density is usually downstream from the maximum *N. mercedis* and *C. franciscorum* densities. This pattern may be attributable, at least in part, to interactions between the caridean shrimp populations. Further study is necessary to clarify the factors responsible for this distribution pattern.

The possible interaction between these two shrimp can be expressed in a series of indices of overlap or crowding. Hurlbert (1978) has recently reviewed overlap indices and defines niche overlap as the degree to which frequency of interspecific encounter is higher or lower than it would be if each species utilized each resource state in proportion to the resource's abundance. For spatial overlap, when each sample unit or "resource state" is considered to be equally abundant Hurlbert's index of interspecific encounter reduces to Lloyd's (1967) index of interspecies patchiness: $L = n\Sigma\,(P_{xi} \cdot P_{yi})$ and refers to the likelihood that two organims will bump into or somehow crowd each other during a given time interval (Hurlbert, 1978). $L = 0$ when no distributional overlap occurs; $L = 1$ when both species are uniformly distributed, and $L > 1$ if each species has a clumped distribution and their distributions tend to coincide.

Spatial overlap (L) was calculated for each collection date in 1977 and 1978 and the results are presented in Table I. L was generally greater than 1.0, indicating a higher probability of interspecific encounter than if both *C. franciscorum* and *P. macrodactylus* were distributed uniformly, *e.g.*, on July I 1978, the probability of interspecific encounter was 3.42× higher than if both species were distributed uniformly. In 1977 spatial overlap was greatest from about August through October, whereas in 1978 spatial overlap was greatest from April through July. Spatial overlap was lower in early 1977 because a large portion of the *P. macrodactylus* population was in the San Joaquin River or in the sloughs bordering Suisun Bay. In 1978 the *P. macrodactylus* catch was high in the sloughs from August to October where no specimens of *C. franciscorum* were collected, thus leading to reduced spatial overlap.

Niche overlap is generally not reciprocal, *i.e.*, species x generally impinges on species y to a different degree than species y impinges on species x (Hurlbert, 1978). Thus, it is of interest to measure directional overlap or mean crowding of species x by species y and vice versa (Lloyd, 1967; Hurlbert, 1978): $Z_{x(y)} = (\Sigma x_i \cdot y_i)/x$. If $Z_{x(y)} > \bar{y}$ then species x has more experience of species y on the average than would be the case if both species were independently distributed. The mean crowding values of *C. franciscorum* by *P. macrodactylus,* etc., are presented as part of Table I. $Z_{x(y)}$ is consistently greater than $\bar{y}$. In 1977 the mean crowding of *Crangon* by *Palaemon*, or the average number of *Palaemon* encountered by an individual *Crangon,* was generally greater than the mean crowding of *Palaemon* by *Crangon*. The reverse was true in 1978.

Competition between these two species may not be occurring even in spite of considerable spatial overlap since space is not likely to be limiting. However, trophic overlap is also great (Siegfried, in preparation) and under some conditions the availability of prey, *i.e., N. mercedis,* could be limiting. If that is the case, competition would have been most intense in 1977 when *N. mercedis* densities were about 15% of normal values (Siegfried *et al.*, 1979). It might be significant that the highest spatial overlap values occurred in the later half of 1977 when *C. franciscorum* and *P. macrodactylus* were both abundant and *N. mercedis* density was very low.

The association between *C. franciscorum* and *P. macrodactylus* is very recent in the San Francisco Bay Estuary. There is no quantitative data available to evaluate the effect, if any, on the introduction of *P. macrodactylus* on native shrimp populations of the delta. *P. macrodactylus* appears more tolerant of some environmental conditions than *C. franciscorum*, occurring not only in the same habitats as *C. franciscorum* but in additional habitats, *e.g.*, in the San Joaquin River and in pilings near shore, not used by *C. franciscorum*. This may provide a competitive advantage during periods in which resources become limiting, perhaps during droughts. Careful management of upstream diversions and water projects in the delta may be required to protect the native shrimp of the delta.

The author wishes to thank the Biological Survey, New York State Education Department, for support during the data-analysis and manuscript-preparation phases of this work.

This work was supported, in part, by a series of gifts to the author and A. W. Knight and the Regents of the University of California from Dow Chemical Company, Pittsburg, CA. I thank J. Orsi and C. Knutson of the California Department of Fish and Game Delta Study Group for providing caridean shrimp samples and *N. mercedis* abundance data. I also thank M. Kopache and B. Louks for their assistance in the field and laboratory. K. Conway and L. Picarazzi, respectively, deserve special thanks for preparation of the figures and typing of several drafts of this manuscript.

Summary

The seasonal abundance and distribution of the native caridean shrimp, *Crangon franciscorum,* and the introduced shrimp, *Palaemon macrodactylus,* in the channel areas of the San Francisco Bay Delta were studied from April, 1977, through October, 1978. *C. franciscorum* reproduces earlier in the year and grows to a larger size than *P. macrodactylus. C. franciscorum* reproduction occurs from December to June in the high salinity waters of San Francisco Bay and the Pacific Ocean. *P. macrodactylus* reproduction occurs from May to September in the delta as well as in higher salinity habitats. Length–weight and length–fecundity relationships differ significantly between the two shrimp. Both shrimp are limited upstream by low salinities, few shrimp occurring at salinities < 1‰. The downstream distribution of these shrimp is related to prey availability, *i.e., Neomysis mercedis* abundance.

Indices of spatial overlap, or interspecies patchiness, indicate a high degree of overlap which varied seasonally and exhibited markedly different patterns in 1977 and 1978. Directional crowding (intraspecific patchiness) also differed between 1977 and 1978. *P. macrodactylus* appears more tolerant of varied environmental conditions than *C. franciscorum,* occurring in the same habitats and also in additional ones not utilized by *C. franciscorum.* This may give *P. macrodactylus* a competitive advantage when trophic resources become limiting.

LITERATURE CITED

Allen, J. A., 1960. On the biology of *Crangon allmani* Kinahan in Northumberland waters. *J. Mar. Biol. Assoc. U.K.*, **39**: 481–508.

Barclay, W., 1978. Development of spawning and mass larva-rearing techniques for the euryhaline shrimp *Palaemon macrodactylus.* Davis, California. Pp. 137–143 in A. W. Knight, Ed., *Studies on Bioenergetics, Osmoregulation, Development, Behavior, and Survival of Several Aquaculture Organisms.* Dep. Land, Air and Water Resources, Water Sci. and Eng. Paper No. 4507, University of California, Davis.

Bonnot, P., 1932. The California shrimp industry. *Calif. Dep. Fish Game Bull.* **38**: 20 pp.

Broekma, M. M., 1941. Seasonal movements and the osmotic behavior of the shrimp *Crangon crangon* L. *Arch. Neerl. Zool.,* **6**: 1–100.

Durkin, J. T., and S. J. Lipovsky, 1977. *Aquatic disposal field investigations, Columbia River disposal site, Oregon. Appendix E. Demersal fish and decapod shellfish studies.* Tech. Rep. D-77-30, Environ. Effects Lab., U. S. Army Eng. Waterways Exp. Sta., Vicksburg, Mass. pp. 119–129.

Ganssle, D., 1966. Fishes and decapods of the San Pablo and Suisun Bays. pp. 64–94 in: D. W. Kelley, Ed., *Ecological Studies of the Sacramento-San Joaquin Estuary.* Calif. Dep. Fish Game Bull., **133**.

Hulbert, S. H., 1978. The measurement of niche overlap and some relatives. *Ecology,* **59**: 67–77.

Isreal, H. R., 1936. A contribution toward the life histories of two California shrimps, *Crago franciscorum* (Stimpson) and *Crago nigracauda* (Stimpson). *Calif. Dep. Fish Game Bull.,* **46**: 1–28.

Khorram, S., and A. W. Knight, 1977. Combined temperature-salinity effects on grass shrimp. *J. Environ. Eng. Div., Am. Soc. Civil Eng.,* **103**: 381–388.

Krygier, E. E., and H. F. Horton, 1975. Distribution, reproduction, and growth of *Crangon nigricuada* and *Crangon franciscorum* in Yaquina Bay, Oregon. *Northwest Sci.*, **49**: 216–240.

Little, G., 1969. The larval development of the shrimp *Palaemon macrodactylus* Rathbun, reared in the laboratory, and the effect of eyestalk extirpation and development. *Crustaceana*, **17**: 69–87.

Lloyd, A. J., and C. M. Yonge, 1947. A study of *Crangon vulgaris* L. in the Bristol Channel and Severn Estuary. *J. Mar. Biol. Assoc. U.K.* **26**: 626–661.

Lloyd, M., 1967. Mean crowding. *J. Anim. Ecol.*, **36**: 1–30.

Maucher, W. D., 1961. Statistische untersuchunger in den körperporotionen wischen der Nord-und Ostee form von *Crangon crangon*. *Kiel. Meeresforsch.*, **17**: 219–227.

Meredith, S. S., 1952. A study of *Crangon vulgaris* in Liverpool Bay area. *Proc. Trans. Liverpool Biol. Soc.*, **1950–1952**: 75–109.

Nelson, S. G., M. A. Simmons, and A. W. Knight, 1979. Ammonia excretion and its relationship to diet in a benthic estuarine shrimp, *Crangon franciscorum* Stimpson (Crustacea: Crangonidae). *Mar. Biol.* **54**: 25–31.

Newman, V. A., 1963. On the introduction of an edible oriental shrimp (Caridea, Palaemonidae) to San Francisco Bay. *Crustaceana*, **5**: 119–132.

Price, K. S., Jr., 1962. Biology of the sand shrimp, *Crangon septemspinosa* in the shore zone of the Delaware Bay region. *Chesapeake Sci.*, **3**: 224–255.

Remane, A., and C. Schlieper, 1971. Biology of brackish water. *Die Binnengewässer*, Vol. 25, 327 pp.

Schmitt, W. L., 1921. *The marine decapod crustacea of California*. Univ. Calif. Publ. Zool., No. 23. 470 pp.

Shaner, S. W., 1978. Osmotic and ionic regulation in the euryhaline shrimp *Crangon franciscorum* (Stimpson) (Crustacea: Natantia). Pp. 106–121 in: A. W. Knight, Ed., *Studies on Bioenergetics, Osmoregulation, Development, Behavior, and Survival of Several Aquaculture Organisms*. Dept. Land, Air and Water Resources, Water Sci. and Eng. Paper No. 4507, Univ. of Calif., Davis, California.

Sharp, J. W., R. M. Sitts, and A. W. Knight, 1978. Effects of Kelthane and temperature on the respiration of *Crangon franciscorum*. *Comp. Biochem. Physiol.*, **59**: 75–79.

Siegfried, C. A., A. W. Knight, and M. E. Kopache, 1978. *Ecological studies on the western Sacramento-San Joaquin Delta during a dry year*. Dept. Land, Air, and Water Resources, Water Sci. and Eng. Paper No. 4506. Univ. of California, Davis. 121 pp.

Siegfried, C. A., M. E. Kopache, and A. W. Knight, 1979. The distribution and abundance of *Neomysis mercedis* in relation to the entrapment zone in the western Sacramento-San Joaquin Delta. *Trans. Am. Fish. Soc.*, **108**: 260–268.

Sitts, R. M., 1978. *Ecological Aspects of the Estuarine Shrimps* Neomysis mercedis, Crangon franciscorum, *and* Palaemon macrodactylus. Ph.D. Dissertation, University California, Davis, California, 79 pp. Diss. Abs. 7905210.

Smith, R. I., and J. T. Carlton, Eds., 1975. *Lights Manual: Intertidal Invertebrates of the Central California Coast*. University California Press, Berkeley, California. 716 pp.

Steel, R. G. D., and J. H. Torrie, 1960. *Principles and Procedures of Statistics*. McGraw Hill, New York. 481 pp.

Reference: *Biol. Bull.*, **159**: 193–205. (August, 1980)

FEEDING OF *NEOMYSIS MERCEDIS* (HOLMES)[1]

CLIFFORD A. SIEGFRIED AND MARK E. KOPACHE

Biological Survey, New York State Education Department, Albany, NY 12230;
and University of Washington, Seattle, WA 95195

The opossum shrimp, *Neomysis mercedis*, is found in brackish to fresh water from Alaska to California, where it is an important component of estuarine food webs. In the San Francisco Bay Delta, it has been shown to be an important food organism for young striped bass (Heubach *et al.*, 1963; Stevens, 1966a), American shad (Stevens, 1966b), white sturgeon (Radtke, 1966), white catfish (Turner, 1966), and the caridean shrimp of the Delta (Siegfried *et al.*, 1978).

Mysids are generally regarded as omnivorous, feeding on detritus, algae, and zooplankton (Bowers and Grossnickle, 1978; Kost and Knight, 1975; Mauchline, 1971; Tattersall and Tattersall, 1951) by virtue of their ability to feed raptorially as well by filtering (Tattersall and Tattersall, 1951). The relative role of each feeding method has received limited attention. Lasenby and Langford (1973) suggested that *Mysis relicta* changes from a benthic detritivore–herbivore during the day to an active predator at night. Richards *et al.*, (1975) postulate that *M. relicta* has been responsible for the decline of large zooplankters in Lake Tahoe, California. Additional studies suggest that copepod predation may be the most important source of food for *M. relicta* in Lake Tahoe (Rybock, 1978; Morgan, 1979). Previous studies of *N. mercedis* have characterized it as omnivorous but made no attempt to determine the relative importance of the various food materials to the total energy intake of the mysid populations (Kost and Knight, 1975; Wilson, 1951).

The only information available indicates that detritus and diatoms are the most abundant items in the gut of *N. mercedis* from the Sacramento-San Joaquin Delta (Kost and Knight, 1975). Crustacean fragments, rotifers, and tintinnids were also found in the gut, but their contribution to the mysids' diet was not examined. Studies of other mysids suggest that they take whatever food is available in their immediate environment (Mauchline, 1967, 1971; Raymont *et al.*, 1964). A recent study of herbivorous feeding by *M. relicta* in Lake Michigan demonstrated algal selection based on size (Bowers and Grossnickle, 1978). Information is lacking on prey selection by *N. mercedis*.

Mysids cultured in the laboratory feed readily on crustaceans such as *Artemia salina* and barnacle nauplii (Foulds and Mann, 1978; Mauchline, 1971). We have held specimens of *N. mercedis* in our laboratories with *Artemia* nauplii as the only food source. Since *N. mercedis* can be an efficient predator in the laboratory, it may also be an important predator in the estuary. This paper evaluates prey selection (both phytoplankton and zooplankton) and the role of carnivory in the nutrition of *N. mercedis* in the Sacramento-San Joaquin Delta.

MATERIALS AND METHODS

The study was conducted as part of a broad baseline investigation of the aquatic resources of the Sacramento River-Suisun Bay portion of the San Francisco Bay-

---

[1] New York State Museum Journal Series No. 283.

Delta Estuary (Seigfried *et al.*, 1978). Sample specimens of *N. mercedis* were collected for gut analysis from two sites at roughly bimonthly intervals from January through November, 1976. One station was located at Chipps Island in eastern Suisun Bay (Station M7), and the other in the Sacramento River channel of the Bay-Delta, about 2 km west of Decker Island (Station M1).

Mysids used for gut-content analysis were selected from samples collected in tows with a #8 plankton net (mesh = 20 μm) and preserved in 5% buffered formalin. Selected for gut-content analysis were specimens of *N. mercedis* of three different sizes: mature mysids 10–11 mm long, immature mysids 7 mm long, and juvenile mysids ≤ 3 mm long (January–August; few found in September–November). Slides were prepared by removing the guts of 8 mature mysids, 10 immature mysids, or 20–25 juvenile mysids; teasing them open, and filtering the pooled contents onto 0.45-μm Millipore filter pads. The entire filter pad was examined at 40× for crustacean or rotifer remains, which were quantified by searching for rami, antennae, or loricas. All phytoplankton prey present on the filter pads were counted and identified from January through August, and perpendicular strips of each filter pad were counted in September and November. The relative amount of each food item was determined by examining 10 microscope fields at 200× and estimating the portion of the field occupied by each item. A Whipple ocular micrometer was used to divide the fields into smaller units for approximating percent coverage.

Prey availability was determined from phytoplankton and zooplankton samples collected at the time of the mysid collections. Phytoplankton samples were obtained from depths of 1, 2, 5, 7, and 9 m and preserved in Lugol solution. Four replicate counts of each phytoplankton sample, with identifications to the generic level, were conducted by the Utermöhl inverted-microscope technique (640×) (Schworbel, 1970). A Whipple ocular micrometer was used to determine cell dimensions. Three replicate zooplankton samples were collected at the same depths as above by a diaphragm-pump method (Herrgesell, 1975) using a #20 plankton net (mesh = 80 μm). Counts were made of the entire sample at 45× in all months except March, when it became necessary to sub-sample for rotifers. A mean for the entire water column was used to estimate prey availability.

Laboratory feeding experiments used *N. mercedis* and *Eurytemora hirundoides* collected in the Delta and held in laboratory culture tanks. Experiments were conducted at 17°C in sea water reconstituted to obtain test water with a salinity of 4‰. Four to 32 individuals of *E. hirundoides* were added to 300 ml of water in each of eight 500-ml flasks. Four flasks were taped to exclude light, and four were clear. One mature unstarved mysid was added to each flask except for one light and one dark "control" flask. Feeding experiments were terminated after 12 hr and the number of copepods remaining in each flask determined. If copepod mortality was significant in the control flask, the results were discarded.

RESULTS

The phytoplankton of the San Francisco Bay Estuary is diverse, with more than 120 species identified (California Department of Water Resources, unpublished). More than sixty genera, primarily diatoms and green algae, were identified in the present study, and more than half of those have been identified in the gut contents of *N. mercedis* (Table I).

TABLE I

Phytoplankton genera identified from samples collected in the Sacramento–San Joaquin Delta Estuary, January–November, 1976. Genera identified in N. mercedis *guts are indicated by* +.

CHRYSOPHYTA

+ *Achnanthes*
Amphipleura
Amphiprora
+ *Amphora*
+ *Asterionella*
Bacillaria
Caloneis
Chaetoceros
+ *Cocconeis*
+ *Coscinodiscus*
+ *Cyclotella*
+ *Cymatapleura*
+ *Cymbella*
+ *Diatoma*
+ *Diploneis*
+ *Epithemia*
+ *Eunotia*
+ *Fragilaria*
+ *Frustulia*
+ *Gomphonema*
+ *Gyrosigma*
Hantzschia
Mallomonas
+ *Melosira*
+ *Meridion*
+ *Navicula*
+ *Nitzschia*
+ *Pinnularia*
+ *Pleurosigma*
+ *Rhoicosphenia*
+ *Rhopalodia*
+ *Skeletonema*
+ *Stauroneis*
+ *Stephanodiscus*
+ *Surirella*
+ *Synedra*
+ *Tabellaria*
Tropidoneis

GREEN ALGAE (CHLOROPHYTA)

Actinastrum
Ankistrodesmus
Chaetophora
Chodatella
Closteriopsis
Closterium
Crucigenia
Golenkiniopsis
Gonium
Microspora
Oocystis
+ *Pediastrum*
Pleurotaenium
Radiofilum
Scenedesmus
Selenastrum
Staurastrum
Ulothrix

DINOFLAGELLATES (PYHRRHOPHYTA)

Glenodinium
+ *Peridinium*

BLUE–GREEN ALGAE (CYANOPHYTA)

Anabaena
Merismopedia
Nodularia
Oscillatoria

The composition of the phytoplankton community changed seasonally during 1976. Various centric diatoms dominated the plankton flora from January through September, and a small blue-green alga, tentatively identified as *Mersmopedia* sp., was dominant in November (Fig. 1), although diatoms continued to dominate the biomass. Phytoplankton density peaked in the spring of 1976 and was lowest in August (Fig. 2). The pattern of phytoplankton population density and composition was similar at both stations.

Zooplankton dynamics within the study area in 1976 were influenced in part by low delta outflows. In January, salinities were low, and the associated fresh-water group of organisms—*Cyclops,* rotifers, and cladocerans—dominated the upstream area (Fig. 3). Rotifers, primarily *Keratella* and *Notholca,* dominated in March. During the remainder of 1976, the fresh-water association was gradually replaced by a brackish-water group dominated by *Eurytemora hirundoides.* By November, a higher-salinity association dominated by *Acartia clausii* and specimens of *Synchaeta* appeared in the study area. Densities reached a maximum in March, coincident with the spring diatom increase. Zooplankton densities were lowest in January. Table II lists the zooplankton collected in the study area in 1976.

Although diatoms were numerically the most abundant prey in the mysid guts, unidentifiable amorphous material and animal fragments generally covered the greatest portion of the filter pads (Fig. 4). Composition of the diet varied in relation to mysid size and location in the estuary. Diatoms composed the greatest percentage of the diet at the upstream site in May. Diatoms accounted for > 50%

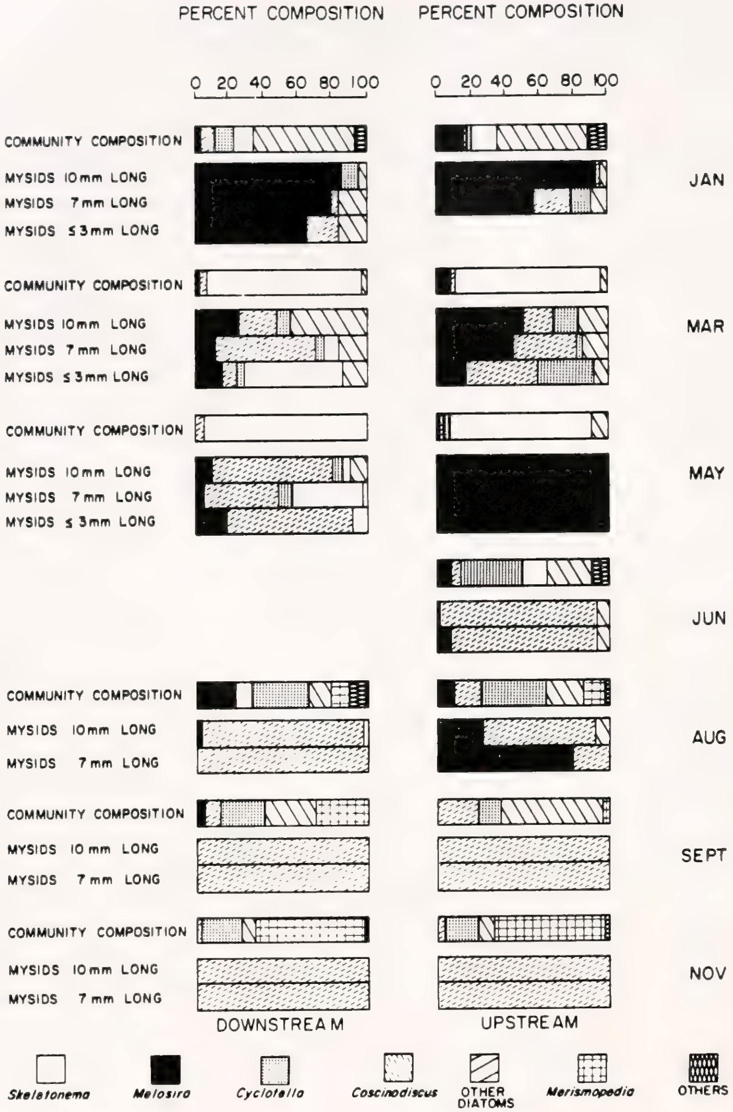


FIGURE 1. Composition of phytoplankton community and phytoplankton consumed by *N. mercedis* in Sacramento-San Joaquin Delta Estuary, January–November, 1976.

of the gut materials on one occasion only, at the upstream site in May. The contribution of diatoms to the diet generally decreased with increasing mysid size.

Crustacean fragments were not found in the guts of mysids ≤ 3 mm long, but rotifers were present in these guts in May (Figs. 3, 4). Animal remains were relatively abundant in the guts of mysids of the larger size classes.

Selective feeding is evident in every month and in all sizes of mysids (Figs. 1, 3). A strong positive selectivity for *Melosira* was evident among all sizes of mysids from January through May. In March and May, although *Skeletonema* was very abundant, accounting for > 90% of the phytoplankton present, it accounted for only a small portion of the diet. From June through November, except for the upstream station in August, *Coscinodiscus* dominated the mysid diet, while all other forms were "negatively selected."

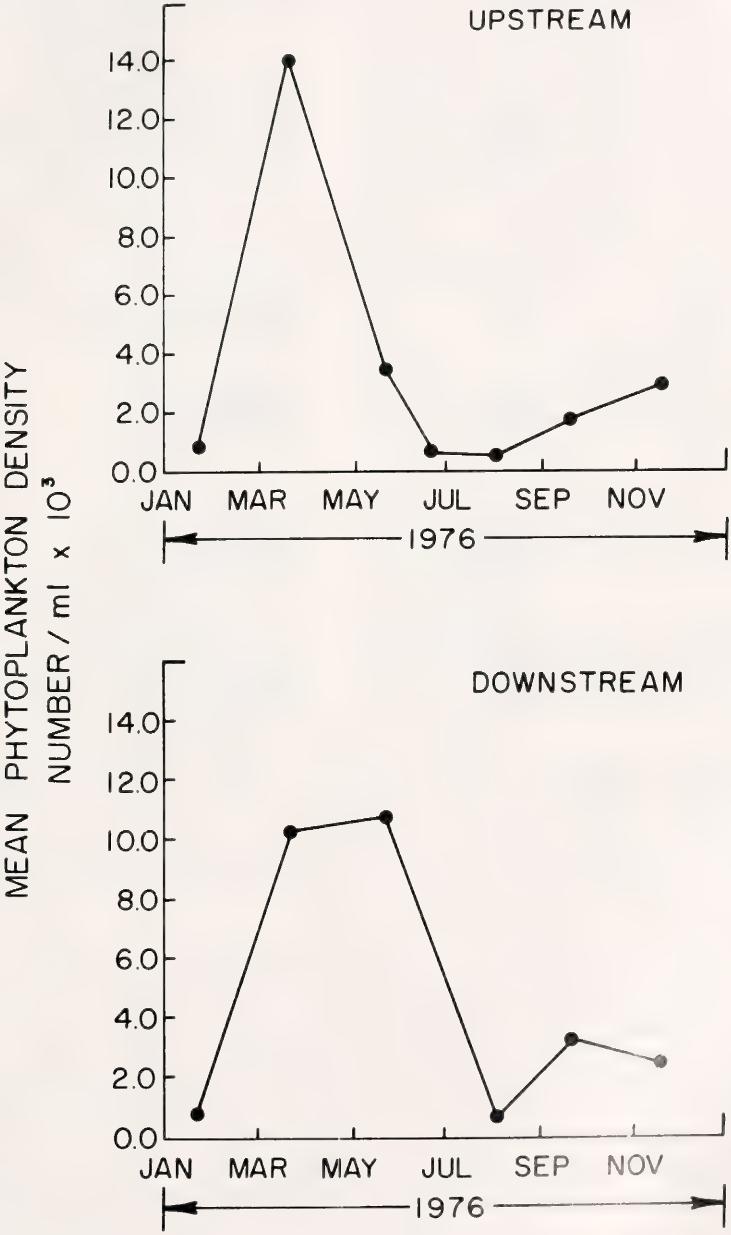


FIGURE 2. Mean density of phytoplankton at upstream (Station M1) and downstream (Station M7) sites in the Sacramento-San Joaquin Delta Estuary, January–November, 1976.

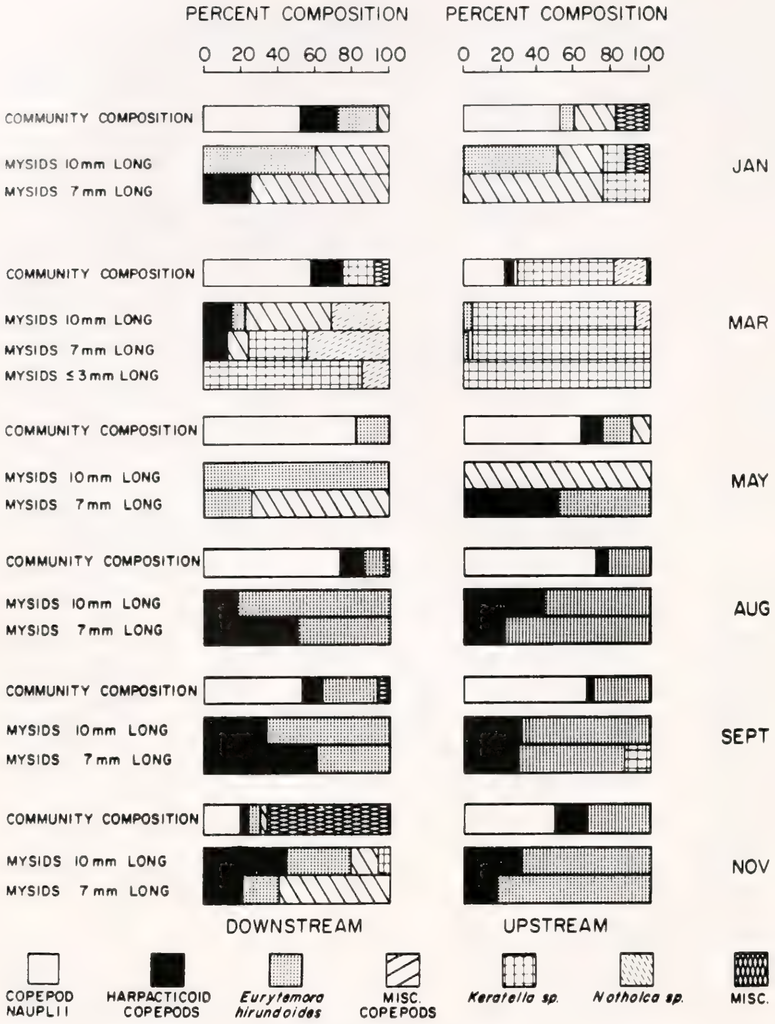


FIGURE 3. Composition of zooplankton community and zooplankton consumed by *N. mercedis* in Sacramento-San Joaquin Delta Estuary, January–November, 1976.

Selection was also evident among mysids feeding on zooplankton. Although copepod nauplii were generally the most abundant crustacean plankters in the system, none were evident in the gut contents of *N. mercedis* (Fig. 3). Rotifers were abundant in March and preyed upon heavily by *N. mercedis*, averaging 29/mysid at the upstream station. *Keratella* and *Notholca* were the rotifers ingested most frequently although additional soft-bodied forms, *i.e.*, *Synchaeta*, may have been ingested but not recognized in the gut contents.

Eurytemora hirundoides and harpacticoid copepods (adult and copepodites) were the crustaceans (Fig. 3) eaten most frequently. Mysids ≥ 7 mm long caught each month except November and March had consumed an average of one to two copepods each. In November and March, the average was three to four copepods in each gut. The largest mysids (≥ 11 mm) always consumed more

TABLE II

Fauna collected in zooplankton samples from the Sacramento-San Joaquin Estuary, January November, 1976.

COPEPODS

- *Eurytemora hirundoides*
- *Acartia clausii*
- *Diaptomous* sp.
- *Cyclops* sp.
- *Ectinosoma* sp.
- *Scottalana* sp.
- undet. Harpacticoid a
- undet. Harpacticoid b

CLADOCERA

- *Bosmina longirostrus*
- *Daphnia laevis*
- *D. pulex*
- *D. schodleri*
- *D. galeata*
- *Monospilus dispar*
- *Diaphanosoma leuchtenbergianum*

ROTIFERA

- *Polyarthra* sp.
- *Kellicottia* sp.
- *Filinia* sp.
- *Synchaeta* sp.
- *Keratella* sp.
- *Notholca* sp.
- *Brachionus* sp.
- *Platyias* sp.
- *Asplanchna* sp.
- *Ascomorpha* sp.
- *Tetrasiphon* sp.
- *Pleurotrocha* sp.
- *Trichotria* sp.
- *Wigrella* sp.

MISCELLANEOUS GROUPS

- *Rhithropanopeus harrisii* (zoea larvae)
- *Balanus* sp. (nauplii)
- *Palaemon macrodactylus* (larvae)

crustaceans than mysids 7 mm long. Juvenile mysids, *i.e.*, ≤ 3 mm long, did not appear to consume crustacean prey.

Laboratory feeding experiments indicate that as copepod density increases, mysid predation also increases (Fig. 5). At low densities, almost no copepods were ingested, whereas at concentrations of 32/300 ml, almost half the copepods were eaten in 12 hr. Results of the field anaylsis are quite variable but indicate generally increased predation with increasing copepod density.

Feeding observations suggest that *N. mercedis* is not a particularly active predator. Mysids position themselves horizontally near the bottom or along the walls of the feeding chambers. Copepods were captured when drawn by feeding currents toward the first pair of thoracic appendages. Copepods were not captured until positioned ventral to the eyestalks, after which the mysid seized and held the copepod with its thoracic endopods while forming a cagelike structure with the remaining thoracic appendages. The prey was positioned below the mouthparts and consumed in its entirety, sometimes anterior first, sometimes posterior first. Copepods were often able to escape from the feeding current before becoming vulnerable. Ingested copepods appear light to dark brown in the guts of live mysids.

Neomysis merecdis appears to feed continuously, with a peak in activity for large mysids during darkness (Fig. 6). The number of copepods consumed per mysid was greater, and the percentage of unrecognizable material less, in larger mysids (≥ 11 mm) collected near midnight than in those collected during daylight. This pattern was not apparent in small mysids. Fine filter feeding appears to be a continuous process, since the number of diatoms per mysid did not vary significantly.

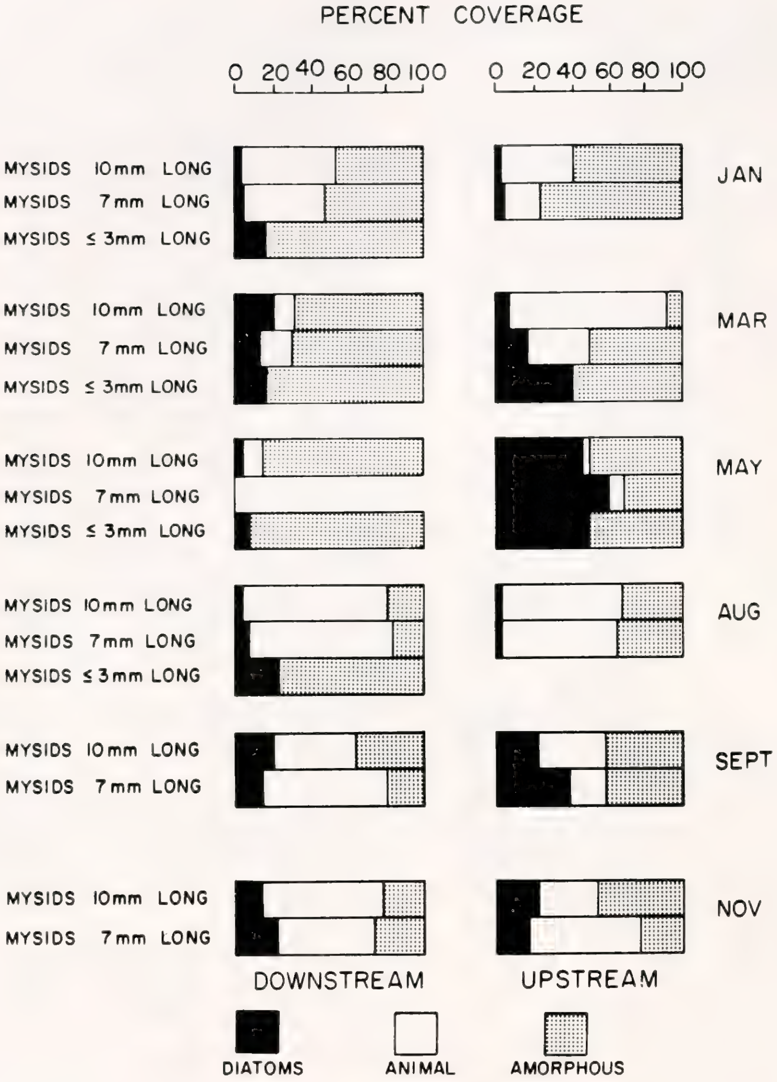


FIGURE 4. Percentage of gut-content filter-pad coverage attributable to phytoplankton, animal material, and amorphous materials, January–November, 1976.

DISCUSSION

Kost and Knight (1975) classified the amorphous material that was the most abundant material in the mysid guts as detritus. Detritus derived from vascular plants and inorganic particulate matter was rarely encountered in the present or previous study. The unidentifiable material present in the mysid guts may be the contents of fragmented algae or crustacean body fluids. Rybock (1978) found considerable "detritus" in the guts of specimens of *Mysis relicta* collected from areas of Lake Tahoe where no "detritus" existed and concluded that this material was well digested and macerated prey. The role of detritus in the nutrition of *Neomysis mercedis* requires further study.

The selectivity patterns of mysids ingesting phytoplankton represent capturability based on size rather than true preference. *Melosira* and *Coscinodiscus*, the most frequently ingested phytoplankters, represent two of the largest algae available, with *Coscinodiscus* occurring as single cells that often exceed 50 μm in diameter and *Melosira* occurring as filaments of relatively large cells. *Skeletonema* occurred

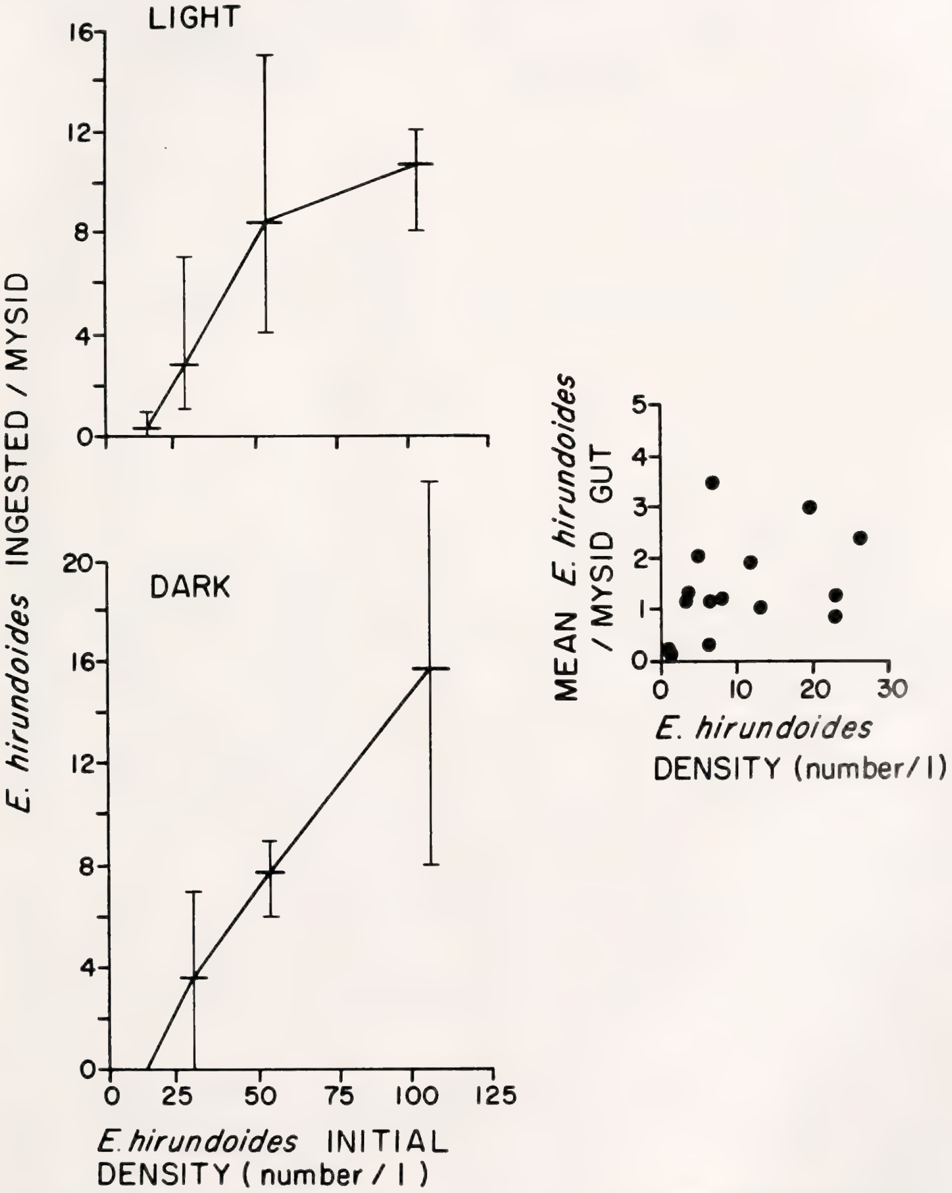


FIGURE 5. Number of specimens of *Eurytemora* ingested by *N. mercedis* in relation to *Eurytemora* density.

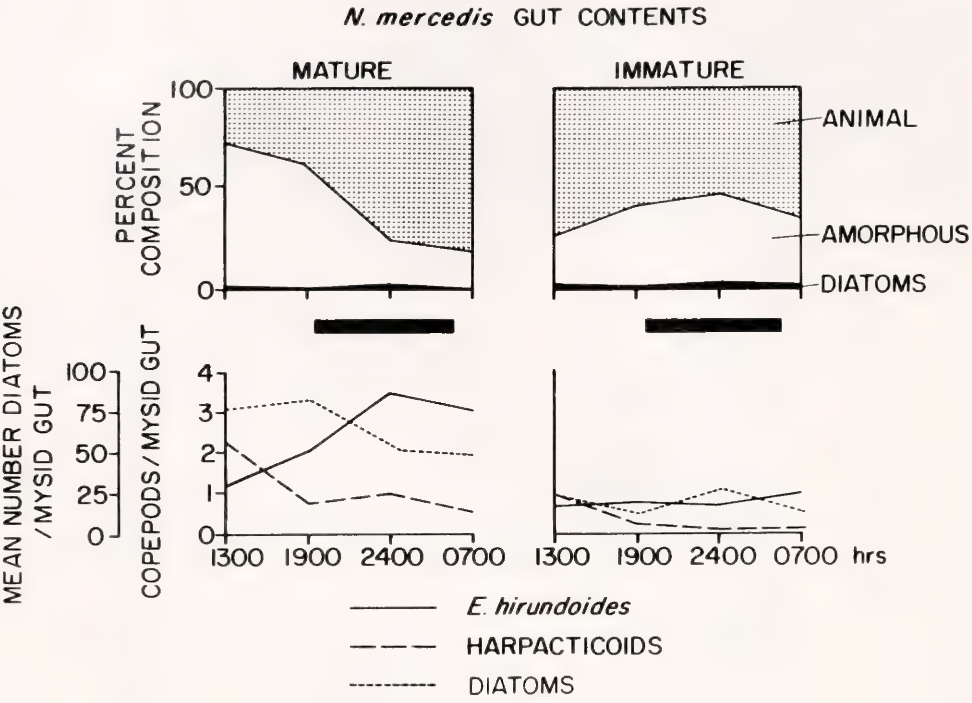


Figure 6. Diel variation in *N. mercedis* gut contents, June 28, 1976.

in short filaments of small cells, generally less than 10 μm long, and in spite of its dominance in the phytoplankton was relatively unimportant in the mysid diet. *Merismopedia*, a small alga, generally occurring in a tetrad less than 5 μm wide, was not identified in the diet of *N. mercedis* although it was very abundant in the fall. Smaller mysids tended to feed on the smaller diatoms as well as the larger forms.

Similar results were reported for mature *M. relicta* from Lake Michigan (Bowers and Grossnickle, 1978). *M. relicta* was found to feed almost exclusively on the largest algal size fraction available, *i.e.*, filamentous diatoms. Algae that passed through a 53-μm mesh were not consumed during *M. relicta* feeding trials. Grazing pressure by mysids on large filamentous diatoms may influence phytoplankton community composition, particularly in the spring.

Peak phytoplankton concentrations were significantly lower in 1976 than in any previous year since records were initiated, *i.e.*, 1966 (Arthur and Ball, 1978). The low phytoplantkon concentrations, believed to be due to the upstream movement of the entrapment zone (Arthur and Ball, 1978), may have been indirectly responsible for the poor success of the mysid population in 1976 (J. Orsi and C. Knutson, Calif. Dept. Fish and Game, unpublished).

The negative selection for copepod nauplii evident in the present study was also evident in Rybock's (1978) study of *M. relicta* in Lake Tahoe. Nauplii are apparently better able to avoid the mysids' feeding currents than are later copepod life stages.

Differences in feeding by various life stages of *N. mercedis* allow efficient partitioning of food resources, thus reducing intraspecific competition. Larger mysids

TABLE III

Caloric value of zooplankton and diatoms in guts of specimens of N. mercedis, *from the San Francisco Bay-Delta Estuary, 22 January–16 November, 1976.*

Date	11 mm mysids		7 mm mysids	
	Zooplankton cal/mysid $\times 10^{-2}$	Phytoplankton cal/mysid $\times 10^{-5}$	Zooplankton cal/mysid $\times 10^{-2}$	Phytoplankton cal/mysid $\times 10^{-2}$
Upstream (Station M1)				
22 Jan	1.1	1.1	0.3	0.7
26 Mar	4.1	1.0	3.3	0.7
26 May	0.6	24.0	0.2	1.2
28 Jun	3.0	1.6	1.3	1.7
02 Aug	1.5	1.0	1.2	1.5
21 Sept	1.6	*	0.7	*
16 Nov	3.0	*	0.7	*
Downstream (Station M7)				
22 Jan	1.0	0.6	0.5	0.8
26 Mar	1.9	0.4	0.4	0.2
26 May	1.2	24.0	0.3	0.4
28 Jun	—	—	—	—
02 Aug	1.9	0.7	0.5	0.4
21 Sept	0.7	*	0.5	*
16 Nov	2.3	*	0.2	*

* Cells fragmented, no estimates of total number of caloric equivalents made.

optimize net energy per unit feeding time by "selecting" the largest prey available Juvenile mysids have a smaller range of prey available.

One of the most interesting aspects of feeding by omnivores is the relative contribution of various trophic levels to nutrition. Mysids feed as herbivores when small, whereas carnivory increases in importance in larger mysids. Mean gut contents were converted to caloric equivalents by empirically determined conversion factors to assess the relative contribution of herbivory and carnivory to ingestion by *N. mercedis* (Table III).

Carnivory accounted for $> 90\%$ of the energy represented by food material present in the guts of mysids ≥ 7 mm (Table III). Mysids would be expected to pass different foods through their guts at different rates and may assimilate them with different efficiencies (Pechén-Finenko and Pavlovskaya, 1973). That could alter the above estimates (Table III) but should not change the overall patterns. The relative role of predation to the nutrition of *M. relicta* in Lake Tahoe is similar (Rybock, 1978).

Phytoplankton populations were very low in the Delta in 1976. That may have contributed to an increased importance of carnivory. However, conversion of gut-content information presented by Kost and Knight (1975) to caloric equivalents (assuming, conservatively, that copepods consumed = 1/10 of crustacean fragments detected) indicates that predation accounted for about 84% of the food energy values. Although herbivory may be of direct importance during blooms (Bowers and (Grossnickle, 1978), predation appears to be the most important feeding mode for *N. mercedis* in the Sacramento–San Joaquin Delta.

This work was supported, in part, by a gift to the senior author, A. W. Knight, and the Regents of the University of California from Dow Chemical Company, Pittsburg, CA. The authors thank A. W. Knight for providing laboratory facilities, A. Hipps and K. Conway for preparation of the figures, and L. Picarazzi for typing of the manuscript. The senior author thanks the Biological Survey, New York State Education Department, for support during manuscript preparation.

SUMMARY

The diet of the opossum shrimp, *Neomysis mercedis,* in the Sacramento River Estuary was studied in relation to food availability, *i.e.*, plankton, from January through November, 1976. The composition of the diet of *N. mercedis* varied in relation to mysid size and prey availability. Mysids exhibited strong positive selection for the large diatom prey species while "avoiding" small diatom prey. Although diatoms were the most abundant prey identified from the guts of specimens of *N. mercedis* it was determined that predation on rotifers and copepods accounted for $> 80\%$ of the energy consumed by other-than-juvenile mysids (≥ 7 mm in length). Juvenile mysids (≤ 3 mm in length) ingested rotifers when rotifers were abundant but were not found to consume copepods. Laboratory feeding experiments indicate a density-dependent feeding by *N. mercedis* on copepods, *i.e.,* as copepod density increases mysid predation on copepods also increases. Feeding observations indicate that *N. mercedis* is not a particularly active predator, capturing prey drawn into its feeding current but not actively pursuing prey. *N. mercedis* appears to feed continuously, with a peak in activity for mature mysids during darkness, a pattern not apparent in immature mysids. Consumption of the detritus was not considered significant. Although herbivory may be of direct importance during the spring diatom increase, carnivory was the major source of energy for *N. mercedis* in the Sacramento River during 1976.

LITERATURE CITED

ARTHUR, J. F., AND M. BALL., 1978. *Entrapment of suspended materials in the San Francisco Bay-Delta Estuary.* United States Department of the Interior, Bureau of Reclamation, Mid-Pacific Region, Sacramento, California. 106 pp.

BOWERS, J. A., AND N. E. GROSSNICKLE, 1978. The herbivorous habits of *Mysis relicta* in Lake Michigan. *Limnol. Oceanogr.,* **23**: 767–776.

FOULDS, J. B., AND K. H. MANN, 1978. Cellulose digestion in *Mysis stenolepis* and its ecological implications. *Limnol. Oceanogr.,* **23**: 760–766.

HERRGESELL, P. L., 1975. *Crustacean zooplankton biomass and its relationship to varying levels of eutrophy in Lake Berryessa, California.* Ph.D. Dissertation, Univ. of Calif., Davis, California, 293 pp. Diss. Abs. 7620991.

HEUBACH, W., R. J. TOTH, AND A. J. MCCREADY, 1963. Food of the young–of–the–year striped bass (*Roccus saxatilis*) in the Sacramento-San Joaquin River System. *Calif. Fish Game,* **49**: 224–239.

KOST, A. L. B., AND A. W. KNIGHT, 1975. The food of *Neomysis mercedis* Holmes in the Sacramento-San Joaquin Estuary. *Calif. Fish Game,* **61**: 35–46.

LASENBY, D. C., AND R. R. LANGFORD, 1973. Feeding and assimilation of *Mysis relicta. Limnol. Oceanogr.,* **18**: 280–285.

MAUCHLINE, J., 1967. The biology of *Schistomysis spiritus* (Crustacea, Mysidacea). *J. Mar. Biol. Assoc. U.K.,* **47**: 383–396.

MAUCHLINE, J., 1971. The biology of *Neomysis integer* (Crustacea: Mysidacea). *J. Mar. Biol. Assoc. U.K.*, **51**: 347–354.

MORGAN, M. D., 1979. Mysis relicta *Population Dynamics in Lake Tahoe.* Ph.D. Dissertation, Univ. of Calif., Davis, Calif.

PECHEN-FINENKO, G. A., AND T. V. PAULOVSKATA, 1973. Comparative importance of detritus and algae in the food of *Neomysis mirabilis. Hydrobiol. J.*, **11**: 28–32.

RADTKE, L. D., 1966. Distribution of smelt, juvenile sturgeon, and starry flounder in the the Sacramento-San Joaquin Delta with observations on food of sturgeon. *Calif. Dept. Fish Game Fish Bull.*, **136**: 115–129.

RAYMONT, J. E. C., J. AUSTIN, AND E. LINFORD, 1964. Biochemical studies on marine zooplankton. I. The biochemical composition of *Neomysis integer. J. Cons. Cons. Intern. Explor. Mer.*, **28**: 354–363.

RICHARDS, R. C., C. R. GOLDMAN, T. C. FRANTZ, AND R. WICKWIRE, 1975. Where have all the *Daphnia* gone? The decline of a major cladoceran in Lake Tahoe, California–Nevada. *Int. Ver. Theor. Angew. Limnol. Verh.*, **19**: 835–842.

RYBOCK, J. T., 1978. Mysis relicta *Lovén in Lake Tahoe: Vertical Distribution and Nocturnal Predation.* Ph.D. Dissertation, University of California, Davis, California. 116 pp. Diss. Abs. 7912972.

SIEGFRIED, C. A., A. W. KNIGHT, AND M. E. KOPACHE, 1978. *Ecological studies on the western Sacramento-San Joaquin Delta during a dry year.* University of California, Davis, Department of Water Science and Engineering Paper 4506, Davis, California. 121 pp.

SITTS, R. M., 1978. *Ecological aspects of the estuarine shrimps* Neomysis mercedis, Crangon franciscorum, *and* Palaemon macrodactylus. Ph.D. dissertation. Unversity of California, Davis, California. 79 pp. Diss. Abs. 7905210.

SCHWORBEL, J., 1970. *Methods of Hydrobiology.* Pergamon Press, New York. 200 pp.

STEVENS, D. E., 1966a. Food habits of striped bass, *Roccus saxatilis*, in the Sacramento-San Joaquin Delta. *Calif. Dep. Fish Game Fish Bull.*, **136**: 68–96.

STEVENS, D. E., 1966b. Distribution and food habits of the American shad, *Alosa sapidissima*, in the Sacramento-San Joaquin Delta. *Calif. Dep. Fish Game Fish Bull.*, **136**: 97–107.

TATTERSALL, W. M., AND O. S. TATTERSALL, 1951. *The British Mysidacea.* Royal Society, London, No. 136. 460 pp.

TURNER, J. L., 1966. Distribution and food habits of Ictalurid fishes in the Sacramento-San Joaquin Delta. *Calif. Dep. Fish Game Fish Bull.*, **136**: 130–143.

WILSON, R. R., 1951. *Distribution, Growth, Feeding Habits, Abundance, Thermal and Salinity Relationship of* Neomysis mercedis *(Holmes) from the Nicomekl and Serpentine Rivers, British Columbia.* M.A. thesis, University of British Columbia, Vancouver, British Columbia. 67 pp. Thesis call no. LE 3B1 1951A8 D5405.

Reference: *Biol. Bull.*, **159**: 206–218. (August, 1980)

MECHANISMS OF COORDINATION BETWEEN MOULTING AND REPRODUCTION IN TERRESTRIAL ISOPOD CRUSTACEA

C. G. H. STEEL

Department of Biology, York University, Downsview, Ontario, M3J 1P3, Canada

It has long been believed that both moulting and egg development in decapod Crustacea are under inhibitory hormonal control by neurosecretions released from the sinus gland (Gabe, 1966; Adiyodi and Adiyodi, 1970; Sochasky, 1973). The observation of apparently synchronous moulting and reproduction in adult crabs (Panouse, 1947) led initially to the view that the processes were "synergistic" and that a single hormone might control both. This view subsequently gave way to the concept of "antagonism" between somatic and reproductive growth, according to which one process occurs only at the expense of the other (Passano, 1960; Charniaux-Cotton and Kleinholz, 1964; Bliss, 1966; Adiyodi and Adiyodi, 1970; Sochasky, 1973). Both concepts developed from consideration of the temporal relations between moulting and reproduction among decapods; their applicability to other groups of the Crustacea is not clear.

The present paper examines the mechanisms which coordinate and regulate reproduction and moulting in terrestrial isopods. In the laboratory, as in the field (Heeley, 1941), these animals moult frequently and regularly and can produce several broods of young per year. Moulting and breeding in decapods maintained in the laboratory are generally less frequent and less regular. Information on control of moulting and reproduction in isopods is based primarily on descriptions of the effects of ablation and transplantation of the protocerebrum (Reidenbach, 1965; Bessé, 1968; Bessé *et al.*, 1969; Mocquard *et al.*, 1971; Bessé and Donady, 1972; Charmantier and Trilles, 1975). The conflicting results reported may be related to lack of information concerning the relationships between these processes in normal animals and the consequent inability to perform surgical manipulations at appropriate times. The present paper examines these relationships in normal isopods and shows that moulting and reproduction are controlled by separate environmental cues and probably separate hormones. However, the relationships are more subtle than terms such as "synergism" and "antagonism" imply; rather, moulting and reproduction are coordinated by specific sensory cues which maintain precise, if complex, temporal relations between them.

Materials and Methods

All of the observations and experiments described have employed *Oniscus asellus* (L.). Many of them have been repeated using *Porcellio spinicornis* (Say), *Cylisticus convexus* (De Geer) and *Tracheoniscus rathkei* (Brandt). The conclusions reported are applicable to all these species; however, quantitative differences were found between species, mainly in the total length of the intermoult cycle. The quantitative data reported are those for *Oniscus asellus*. The experiments reported for *Oniscus* have been repeated, and have used both animals born and raised in laboratory cultures and animals collected from the field in early spring before either moulting or reproduction has commenced. All animals used in experiments had a head capsule width of 2.0–2.5 mm. Animals this size in the field are 1-year-olds which have not reproduced (McQueen, 1976). Head capsule width appears to be the most reliable convenient parameter of age (Standen, 1970). Body weight was not used, as terrestrial isopods undergo substantial changes in water content (Lindqvist, 1972; Mayes and Holdich, 1975).

Laboratory cultures were maintained at 21 ± 1°C in a 16L:8D daylength cycle. An individual culture consisted of 6–20 animals in a plastic Petri dish about 9 by 2.5 cm equipped with a humidity wick, crushed limestone from the vicinity of the field collections, and ground Purina rabbit chow, which Merriam (1971) found to provide an optimum diet. Females incubating broods of young were isolated in single culture dishes until the young were liberated. The adult was then removed and the young raised together in the dish. Thus the age of laboratory-born animals was known precisely.

The procedure for determining the various stages of the moult cycle will be described in detail elsewhere (Steel, in prep.). In brief, the sternites of pereion segments 1–4 undergo systematic changes in appearance due to the accumulation of fat cells and calcium deposits, which develop through a characteristic sequence of changes in shape and size at specific times during the cycle. Fourteen distinct configurations are recognizable on brief visual inspection of the animal's ventral surface. Stages 1 through 9 represent premoult. The same 14 stages occur with the same relative durations in all species examined, despite species variation in total cycle length. These stages are correlated with the changes associated with secretion and resorption of the exoskeleton (unpublished observations) and are readily related to the alphabetical subdivisions of the moult cycle used in decapods (Drach, 1939). Being the more familiar, the latter terminology is used in this text. The term premoult is used to refer to the period between moult initiation and ecdysis (Stage D in the terminology of Drach). Postmoult is the period of post-ecdysial secretion and calcification of the exoskeleton (Stages A, B, and C_1–C_3) and intermoult is the period between the end of postmoult and initiation of the next premoult (Stage C_4). Durations given in the text are means ± S.D.

RESULTS

Differential effects of temperature and daylength

Observations on field populations have shown that moulting occurs throughout the summer, whereas females incubating broods of eggs are found only during a part of this season (see McQueen, 1976, for details). These observations imply distinct seasons for each process. This in turn suggests separate environmental regulation of moulting and reproduction. This possibility was examined in the laboratory using both field-collected and laboratory-raised animals of the same size. Females from both sources moulted regularly at intervals of 28 days at 21 ± 1°C. Moulting in males was slightly less frequent and less regular (see Table II). Moulting frequency exhibited a Q_{10} of two between 12° and 22°C and was arrested completely at 5°C. Animals maintained in intermoult at 4°C could be induced to moult by transferrance to 21°C. In such animals, the first sign of early premoult occurs 14 ± 1.9 days after transfer to 21°C. Animals brought in from the field in either spring or autumn from outside temperatures of 0–5°C responded to this increase in temperature in the same way. These observations suggest that premoult is initiated following a threshold number of warm days. Parallel cultures maintained under "short" (8L:16D) or "long" (16L:8D) daylength regimes continue

TABLE I

Effect of daylength on induction of breeding in female Oniscus. *In long days the 17% which did not breed at the first moult all did so at the second. N = 40. See text for details.*

Daylength regime	Long days 16L:8D		Short days 8L:16D	
Type of moult	Breeding	Non-breeding	Breeding	Non-breeding
First moult	83%	17%	9%	91%
Second moult	73%	27%	27%	73%

to moult throughout the year with the same frequency (except under conditions which stimulate vitellogenesis, as described below). Thus, moulting is strongly temperature dependent but is apparently independent of daylength.

Adult females are capable of two different types of moults. One is similar to that of males and no change in form occurs. The other occurs only during the breeding season in the field and is morphogenetic in the sense that the oostegites of pereion segments two to five enlarge enormously to form a brood pouch in which eggs are incubated ("maternal" moult of Heeley, 1941). The influence of daylength on the morphogenetic character of the moult was examined in the following experiment. Twenty female *Oniscus* which had previously been held in intermoult at 4°C were induced to commence moulting by transferrance to 21°C. Half were placed in 8L:16D and half in 16L:8D. A further 20 intermoult females collected from the field in early spring were also set up under these two conditions. The proportions forming brood pouches at each of the first two moults are shown in Table I. No differences between laboratory-bred and field-collected animals were detected. Accordingly, Table I presents the combined data for all 40 animals in each regime. In long days, almost all animals formed brood pouches at the first moult, and of those that did not, all did so at the second moult. Accordingly, the 27% of 40 animals which underwent a non-breeding second moult in long days had all undergone breeding at the first moult. The majority of animals in long days underwent two breeding moults in succession. Conversely, breeding was rare in the short-day animals, even after two moults. Thus, while the occurrence of moulting appears to be independent of daylength, the morphogenetic character of the moult is strongly influenced by daylength.

The interaction of temperature with daylength in *Oniscus* has been discussed in detail by McQueen and Steel (1980). It is sufficient here to note that temperature does not directly affect whether or not reproduction will occur; the role of temperature in regulating reproduction appears to be confined to determining the rapidity with which animals respond to those daylengths which promote reproduction due to the influence of temperature on the length of time between ecdyses. Thus, temperature affects moulting but has no direct effect on reproduction. Daylength and temperature, therefore, exert differential effects on moulting and reproduction.

Breeding cultures can therefore be maintained in the laboratory at 21°C in "long" days. Although moulting continues throughout the year under these conditions, females do not breed throughout the year. These laboratory cultures cease breeding in September in synchrony with field populations, indicating that the cessation of breeding is brought about by different cues from those which induce it. For the following 3 months females remain refractory to the stimulation of reproduction by long daylengths, but become responsive to them again in December, when field populations are completely dormant. Thus, there appears to be a seasonal periodicity in responsiveness to conditions promoting breeding. The absence of such periodicity influencing moulting further supports the view that moulting and reproduction are controlled by separate mechanisms which are differentially influenced by environmental factors.

Stages of oogenesis

Mature eggs are deposited into the brood pouch a few hours after ecdysis (Nemec, 1896). The number of eggs per brood pouch averaged 38 ± 6. In order to examine the interrelations between the development of these eggs and the moulting cycle, it is necessary to recognize the different stages of egg development. The paired ovaries are tubular structures extending throughout the pereion. In non-breeding animals, each ovary contains 19 ± 8 small white oocytes 100–200 μm (mean 131 μm) in diameter. These correspond in size and appearance to the "previtellogenic" oocytes of the amphipod *Orchestia gammarella* (Charniaux-Cotton, 1973). Thus, each animal contains sufficient previtellogenic oocytes for the production of exactly one brood of young without cell division. These oocytes do not change in size, appearance, or number during moult cycles which do not lead to formation of a brood pouch. It therefore seems that oocyte development is arrested in the "previtellogenic" stage until reproduction is stimulated.

When reproduction is stimulated, all the previtellogenic oocytes grow synchronously from 200 to 600 μm. While this is occurring they become progressively more yellow. Globules accumulate in the cytoplasm and increase in size and number with growth of the oocytes. These changes in oocyte size and appearance are commonly regarded as due to the accumulation of yolk. Hence, this phase of oocyte development represents vitellogenesis. That vitellogenesis does indeed occur in *Oniscus* oocytes at this stage is confirmed by the finding that developing oocytes first acquire immunoreactivity to antiserum raised against purified *Oniscus* yolk proteins at a size of 150–200 μm (C.G.H. Steel and R. A. Baron, unpublished observations). Mature eggs newly deposited into the brood pouch are similar in size and appearance to the largest seen in the ovaries.

During the incubation of a batch of mature eggs in the brood pouch, oogonia proliferate and previtellogenic growth of oocytes occurs, thereby restoring the complement of previtellogenic oocytes by the time the brood is liberated. Brood

TABLE II

Modification of chronology of moult cycle by egg development and incubation. The postmoult period lasts 2–3 days. Hence intermoult is considered here to begin 2 days after anterior ecdysis and to end with initiation of premoult. Thus, brood incubation time equals length of intermoult plus postmoult.

	Length of premoult	Length of next intermoult	No. of animals
Males	16.3 ± 2.0 days	10.0 ± 5.1 days	25
Females, non-breeding	16.2 ± 1.8	7.1 ± 1.4	29
Females, first brood	33.0 ± 3.3	32.0 ± 2.6	25
Females, second and subsequent broods	17.7 ± 3.1	33.8 ± 3.7	31

incubation lasts until the young hatch within the brood pouch and crawl out of it. At 21°C the average duration of brood incubation is about 34 days (see Table II), longer than a complete intermoult cycle in non-breeding animals. The mechanisms by which growth of the oocytes and brood incubation are coordinated with moulting activities are examined below.

Effects of reproduction on moulting

As noted above, brood incubation takes longer than a complete intermoult cycle of non-breeding animals. This implies that the chronology of the intermoult cycle is modified during brood incubation.

Since deposition of mature eggs into the brood pouch occurs shortly after ecdysis, incubation commences during the postmoult period. Females incubating eggs were inspected every 3 days for signs of premoult. No premoult was initiated until the end of brood incubation (see also below). This confirms that brood incubation occurs during the intermoult stage (Stage C_4 of Drach, 1939). However, the postmoult and intermoult stages of non-breeding females together last only about 9 days (Table II), whereas, incubation lasts about 34 days at the same temperature. Therefore, initiation of premoult must be delayed about 25 days in females incubating a brood. Thus, brood incubation influences the moult cycle by causing an extension of intermoult from 7 to 32 days (Table II).

Previtellogenic growth of the oocytes occurs during brood incubation, *i.e.*, during postmoult and intermoult. These oocytes undergo fertilization and incubation following a subsequent ecdysis and formation of another brood pouch. Thus, at least one moult occurs between previtellogenic growth and fertilization. This arrangement contrasts with that in higher decapods in which both egg maturation and incubation occur within a single intermoult (Adiyodi and Adiyodi, 1970).

Reproduction also modifies the chronology of premoult. This period lasts 16 days in non-breeding *Oniscus* females at 21°C (Table II). However, in animals kept in intermoult at 4°C, and then transferred to 21°C and long days to stimulate a maternal moult, the premoult period lasts 33 days (Table II). All the constituent sub-stages were found to be of their normal length except for Stage 3 (late D_0 in the nomenclature of Drach, 1939) which was extended from 3 to 20 days in duration. Thus, there is a lengthy pause in the early premoult of animals stimulated to undergo a first maternal moult. Dissection of the ovaries of animals which had been in this protracted late D_0 for various known lengths of time revealed that the changes in size and appearance of the occytes which accompany vitellogenesis commence during this period. Moreover, the oocytes first develop immunoreactivity to anti-vitellogenin serum at this stage (C. G. H. Steel and R. A. Baron, unpublished observations).

Thus, vitellogenesis commences in early premoult, at which time the moulting process undergoes suspension for 16 days, at the end of which premoult changes resume with the same chronology as in non-breeding females. However, vitellogenesis is not completed during the period of premoult suspension. Dissection of ovaries from females at various stages of premoult showed that oocyte growth is not completed until shortly before ecdysis (Table III) and most of the increase in size and accumulation of yellow globules in the oocytes occurs after the premoult has resumed. Suspension of premoult is therefore not necessary throughout vitellogenesis.

Table III

Chronology of vitellogenesis in first and second maternal moult cycles. The oocyte size ranges given indicate the average size increases observed between the beginning and end of the moult stage indicated. Oocyte sizes were measured for 30 oocytes (15 from each ovary) from each animal. "n" gives the number of animals dissected at each stage.

Stage of moult cycle	First maternal moult				Second maternal moult			
	n	Oocyte size range	Oocyte appearance	Stage duration	n	Oocyte size range	Oocyte appearance	Stage duration
Intermoult								
Early	11	100–200 μ	Clear, white	7 days	17	100–200 μ	Clear, white	24 days
Late					19	200–300 μ	Opaque, yellowish	8 days
Premoult								
Early (D_0)	12	200–250 μ	Opaque, yellowish	24 days	12	330 μ	Opaque, yellowish	8 days
Late (D_1–D_3)	16	250–600 μ	Opaque, yellow	9 days	8	330–600 μ	Opaque, yellow	9 days

A further illustration of interactions between reproduction and moulting is seen when females incubating broods of eggs are maintained in environments promoting reproduction, such that two or more maternal moults occur in succession, a new brood pouch being formed under the old one during the premoult following liberation of the brood. Under these conditions, the pause in late D_0 seen in the first maternal moults is absent (Table III). The chronology of all stages of premoult is the same as in non-breeding females. Dissection of the ovaries of these animals revealed that at the beginning of premoult, the oocytes had already developed to an average size of 330 μm (Table III) and had accumulated significant numbers of yellow globules. This indicates that the timing of vitellogenesis in the first maternal moult is different from that seen in subsequent maternal moults. During incubation of the first brood, the oocytes do not cease growth at the end of the previtellogenic stage, but continue into vitellogenesis, such that by the time the brood was liberated the oocytes were about halfway through the changes in size and appearance associated with vitellogenesis (Table III). Thus, the premoult following brood liberation commences with the oocytes already undergoing vitellogenesis. However, vitellogenesis is completed in late premoult, as in first maternal moults. In other words, the later part of vitellogenesis invariably occurs in late premoult, whereas the first part can occur either in early premoult (first maternal moult) or during intermoult (second and subsequent maternal moults). Whichever stage vitellogenesis commences in undergoes a considerable extension relative to its duration in non-breeding animals; the extension to intermoult during incubation is 25 days and the extension to early premoult seen in first maternal moults is 16 days. Thus, the total time occupied by vitellogenesis is similar in both first and subsequent maternal moult cycles, but the position of the first part of vitellogensis in the moult cycle is flexible.

Stimulus to premoult initiation

The finding that intermolt is protracted during incubation suggested the possibility that the timing of the initiation of the next premoult might be regulated by some event associated with incubation. Subsequent to fertilization of the eggs, the role of the mother in brood incubation seems to be confined to production of a watery fluid which surrounds the eggs in the brood pouch, for fertilized eggs can be grown *in vitro* in saline (Sutton, 1972). The eventual liberation of the young from the pouch "seems to be simply a matter of the young crawling out when they are ready" (Sutton, 1972), over a period of up to 2 days (Heeley, 1941). The oostegites are held in a distended position by the brood, but collapse to the sternites when the young are liberated. The first visible signs of premoult are seen 3.1 ± 0.8 days after the complete collapse of the brood pouch.

Premature deflation of the brood pouch was produced by rinsing away the developing eggs or embryos in a fine stream of tap water. This resulted in moult initiation, premoult becoming visible on the third day following collapse of the brood pouch as in normal animals. If one or two eggs remained stuck in the pouch, the appearance of premoult was greatly delayed, usually by about 10 days. Thus, the presence of eggs in the brood pouch is necessary to maintain the intermoult condition. In the converse experiment, the brood pouch of females which had just liberated young was stuffed with cellulose sponge moistened with the brood pouch saline of Sutton (1972). No initiation of premoult was seen in these animals. When the sponge was removed after about 10 days, premoult was detectable after 3 days as if a normal brood had been liberated.

The initiation of premoult is therefore determined by the time of collapse of the oostegites. Presumably, proprioceptive or tactile information from these appendages provides a nervous pathway for regulating release of the moult-controlling hormones. These observations illustrate quite strikingly that events associated with reproduction provide cues which regulate both length and timing of the moult cycle.

However, in males and in non-breeding females, premoult initiation must be accomplished by a mechanism different from the above. The role of a threshold number of warm days has been mentioned earlier. However, the length of intermoult is remarkably constant, especially in females, even after months of constant temperature and daylength. Even nutritional state seems not to be fundamental, for starved animals continue to moult frequently even though they become smaller at each moult. It is clear that moulting does not occur solely to permit growth. However, the mechanism of regular premoult initiation in a constant environment remains unknown.

DISCUSSION

Environmental regulatory factors. Both temperature and daylength influence moulting and reproduction, but have differential effects on the two processes. Moulting is strongly temperature dependent but is unaffected by daylength (McQueen and Steel, 1980). Since moulting ceases at 5°–10°C, moulting in field populations should occur when temperatures reliably exceed 5°–10°C. Local meteorological records show such field temperatures between March and October. Conversely, reproduction is induced by long days (16L:8D) and suppressed by short (8L:16D) in regularly moulting animals. This effect appears to be a conventional photoperiodic response with a critical daylength of about 11.5 hr (McQueen and Steel, 1980). At 44°N, daylength would promote reproduction in the field between April and September. These expectations are confirmed by field observations on natural populations of *Porcellio* in the same vicinity (McQueen, 1976) and of *Oniscus* in England (Heeley, 1941). Thus, daylength confines reproduction to the central portion of the season when temperature permits moulting. It is concluded that moulting and reproduction are confined to their respective seasons by differential responsiveness of the two processes to the environmental cues of temperature and daylength. Such differential responsiveness further suggests the existence of separate physiological regulatory mechanisms for each process, as discussed below.

Superimposed on these effects of temperature and daylength is a seasonal periodicity in the responsiveness of females to environments which promote breeding. Females become refractory to long-day stimulation in the laboratory at about the time when breeding ceases in the field, and remain so for the following 3 months. Similar events occur in cultures of *Porcellio* in France (Bessé and Maissiat, 1971). This refractoriness suggests the intervention of a phenomenon analogous to reproductive diapause. The absence of seasonal periodicity in moulting in the laboratory supports the above inference that moulting and reproduction are separately controlled.

Stimulus to moult initiation. Brood incubation occurs during an intermoult which is prolonged to about four times its duration in non-breeding moult cycles; initiation of the next premoult is delayed until the young are liberated from the brood pouch. The collapse of the brood pouch when the brood is liberated provides a trigger which initiates premoult. Three days elapse before the appearance of the first morphological signs of premoult. Since morphological changes result from prior hormonal changes, it must be in this period between the stimulus and the appearance of morphological changes that the endocrine events associated with premoult are initiated.

All other descriptions of stages of moult cycles in Crustacea rely on the appearance of some tissue change to define the beginning of premoult. Such descriptions of premoult in terms of effects rather than causes inevitably overlook the initial stage(s) of premoult in which key physiological changes occur. Thus, animals would be inappropriately classified as in intermoult. This may account for the high individual variation in indices of metabolic activity noted previously in ostensibly intermoult animals (*e.g.*, Andrieux, 1979). The proportion of the total premoult period which passes without morphological change in *Oniscus* is 3 out of 16 days at 21°C, or 19%. However, most other crustacean moult staging schemes recognize premoult by the occurrence of apolysis in the integument (Drach and Tchernigovtzeff, 1967). In *Oniscus*, apolysis occurs in different regions of the integument between 5 and 8 days after the moult-initiating stimulus (unpublished observations). Thus, if premoult were recognized by apolysis in *Oniscus*, as it is in *Sphaeroma* (Tchernigovtzeff and Ragage-Willigens, 1968), the proportion of the premoult period which would pass unnoticed would increase to as much as 50%.

It is inferred that the stimulus which initiates premoult and the primary hormonal changes occurs before tissue changes become evident, and certainly well before apolysis. Thus, premoult begins well before it is recognizable by conventional moult-stage schemes. This conclusion lends substance to that of Passano (1960), who presumed that early D_0 would not be morphologically detectable, but differs strikingly from that of Reaka (1975) and Vranckx and Durliat (1978), who propose that initiation of premoult may not occur until *after* apolysis.

Coordination of moulting and reproduction. The mechanism discussed above, whereby intermoult is extended during incubation, is but one of several mechanisms which coordinate the moult cycle with reproductive events.

The point in the moult cycle at which vitellogenesis commences was found to be flexible. During a first maternal moult it commences in late D_0 and is accompanied by a temporary suspension of this stage of premoult for 16 days, resulting in a premoult period of double the normal length. In animals in which a second maternal moult follows the first, this prolongation of premoult is not seen and vitellogenesis commences during the preceding intermoult. Thus, different temporal relations are found between vitellogenesis and the moult cycle under different conditions, which again suggests that separate mechanisms control these processes. However, regardless of the moult stage at which vitellogenesis commences, most of the growth in oocyte size occurs during late premoult. Thus, two distinct phases of vitellogenesis can be distinguished: The first phase is flexible in timing and can occur either in intermoult or during a protracted D_0; the second phase invariably occurs during late premoult. The occurrence of vitellogenesis during both intermoult and premoult also occurs in the amphipod *Orchestia* (Charniaux-Cotton, 1973; Meusy *et al.*, 1974), in which this arrangement has been ascribed to the short duration of intermoult relative to premoult (Adiyodi, 1978). However, vitellogenesis in isopods always continues into premoult even when intermoult is prolonged fourfold. This is suggestive less of insufficient time for the completion of vitellogenesis during intermoult than of different physiological requirements for the completion of the two phases.

The fact that a pause in late D_0 is seen only in the first maternal moult suggests that it is not a direct response to the occurrence of vitellogenesis but rather a response to the morphogenetic character of the moult. During a first maternal moult, the epidermis must become reorganized so that a brood pouch will be differentiated. Obviously, this reorganization must be completed before the commencement of cuticle deposition, *i.e.*, prior to the end of D_0. Thus, the protraction of D_0 may be required to effect this reorganization of epidermal cells for differentiation of a brood pouch. In contrast, during a second maternal moult, the first brood pouch is replaced by a second pouch and consequently no epidermal reorganization is required.

Synergism and antagonism. The relations between moulting and reproduction in crustaceans have been widely described as illustrating either "synergism" or "antagonism" between the two phenomena (references in Introduction). These two concepts are based almost entirely on the observation of either simultaneous or consecutive occurrence of moulting and reproduction in decapods. However, this terminology strongly implies that the temporal relations observed between moulting and reproduction are not merely circumstantial, but are a product of specific processes of mutual stimulation or inhibition. There is no compelling evidence of such processes, even in decapods. The present work suggests that this terminology is inappropriate, at least for isopods, and its implications potentially misleading.

The finding that moulting may occur either with or without reproduction according to daylength suggests that the two processes are independent but coordinated responses to environmental cues and are not simply "synergetic" or "antagonistic." Thus, the occurrence of vitellogenesis during premoult in isopods is not interpreted as evidence of "synergism" between reproduction and moulting since there is no evidence that the occurrence of either process stimulates the other. The term "synchrony" as used to describe this phenomenon in *Orchestia* (Meusy *et al.*, 1977) has fewer functional implications and is considered preferable to "synergism."

Adiyodi and Adiyodi (1970) suggest that "antagonism" characterizes the reproductive period in decapods, on the supposition that the demands of the gonad for metabolic reserves interfere temporarily with the growth of the integument. While the pause observed in early premoult of isopods during which vitellogenesis commences could be construed as illustrating this notion, most of the oocyte growth was found to occur in late premoult when cuticle changes are proceeding concurrently. Hence, it is not the metabolic demands of the ovary which produce the pause in premoult. Similarly, the prolongation of intermoult during brood incubation could also be construed as "antagonism." However, there is no need to postulate an inhibition of moulting at this time; the initiation of premoult by sensory input from the brood pouch indicates coordination rather than "antagonism" between moult initiation and brood incubation.

Implications for hormonal control. Substantial evidence exists for many crustacean species of inhibitory neurohormonal control by the brain of both moulting and reproduction (reviewed by Gabe, 1966; Adiyodi and Adiyodi, 1970; Sochasky, 1973). There is continued debate over whether separate moult- and gonad-inhibiting hormones (MIH and GIH) occur or whether both processes are regulated by a single "growth inhibiting principle" (Panouse, 1947). As in decapods, there are conflicting reports concerning the effects of brain lesions on moulting and reproduction in isopods, (reference in Introduction). The present report is the first to examine the mechanisms coordinating moulting and reproduction in normal isopods. Certain requirements of any concept of hormonal control of these processes may now be inferred.

First, separate moult- and gonad-regulating hormones are necessary. All the above arguments that moulting and reproduction are separate but coordinated processes imply separate hormonal mechanisms; it is not possible to explain the interactions found between these processes in terms of a single "growth inhibitory principle." This conclusion is further supported by the finding that one of the groups of neurosecretory cells in the brain of *Oniscus* undergoes cytological changes correlated with the moult cycle while those of another group are correlated with vitellogenesis (unpublished observations).

Second, more than one hormone seems to be involved in vitellogenesis. Ecdysone appears to be necessary for vitellogenesis in *Porcellio* (Bessé and Maissiat, 1971). The present finding that vitellogenesis is always completed in late premoult, when whole body content of ecdysteroids peaks in other isopod species (Charmantier *et al.*, 1976; Hoarau and Hirn, 1978) suggests that ecdysone may be necessary for the completion of vitellogenesis. However, it is highly improbable that ecdysone initiates vitellogenesis, since it may commence either in intermoult or early premoult. Rather, its initiation may be controlled by some other principle (GIH or analogous factor), perhaps acting to stimulate production of vitellogenins. Regulation of the release of this principle by daylength would explain the observed effects of daylength on reproduction. The role of ecdysone might then be in the subsequent uptake of vitellogenins by the oocytes, as has been suggested in *Orchestia* (Meusy *et al.*, 1977; Blanchet *et al.*, 1979). The regulation of vitellogenesis seems to be different in isopods and decapods, for the whole of vitellogenesis usually occurs during intermoult in the latter (Adiyodi and Adiyodi, 1970, for review).

Third, support is given to the proposal that isopod ovaries containing vitellogenic oocytes produce an "ovary hormone" which induces differentiation of a brood pouch (Legrand, 1955; Balesdent, 1965). During first maternal moults, the pause in late D_0 coincides with the beginning of vitellogenesis and ends with apolysis. Secretion of an "ovary hormone" would therefore commence at exactly the appropriate time to elicit the reorganization of the epidermis to form a brood pouch under the influence of ecdysone later in premoult. During second maternal moults, the ovaries contain vitellogenic oocytes at the commencement of premoult, enabling prompt secretion of hormone to ensure that the epidermis retained its ability to differentiate another brood pouch.

This work was supported by a National Research Council of Canada negotiated development grant to York University.

SUMMARY

In terrestrial isopods, different sensory cues initiate reproduction and moulting, indicating that the two processes are controlled by different physiological mechanisms. A specific sensory trigger which initiates premoult is identified; it occurs well before conventional signs of premoult become evident. Specific coordinating mechanisms adjust the chronology of moulting and vitellogenesis under conditions promoting both processes. The first phase of vitellogenesis can occur either in intermoult or early premoult according to conditions and is considered to be independent of ecdysone. The second phase invariably occurs in late premoult and may be ecdysone-dependent. The relations between moulting and reproduction are regarded as separately controlled processes which interact via specific cues which coordinate and adjust the timing of the two processes. Implications of this concept are discussed.

LITERATURE CITED

ADIYODI, K. G., AND R. G. ADIYODI, 1970. Endocrine control of reproduction in decapod Crustacea. *Biol. Rev.*, **45**: 121–165.

ADIYODI, R. G., 1978. Endocrine control of ovarian function in crustaceans. Pages 25–28 in P. J. Gaillard and H. H. Boer, Eds., *Comparative Endocrinology*, Elsevier/North-Holland, Amsterdam.

ANDRIEUX, N., 1979. L'apolyse au cours du cycle d'intermue de deux Crustacés Décapodes Brachyures *Carcinas maenas* Linné et *Carcinas mediterraneus* Czerniavsky. *C. R. Acad. Sci. Paris*, **288**: 1595–1597.

BALESDENT, M. L., 1965. Recherches sur la sexualité et le déterminisme des caractères sexuels d'*Asellus aquaticus Linné* (Crustacé, Isopode). *Bull. Acad. Soc. Lorraines Sci.*, **5**: 1–231.

BESSÉ, G., 1968. Contribution at l'étude du contrôle neurohumoral de la maturation ovarienne et de la mue parturielle chez l'Oniscoïde *Porcellio dilatatus* Brandt. *C.R. Acad. Sci. Paris*, **266**: 917–919.

BESSÉ, G., P. JUCHAULT, J.-J. LEGRAND, AND J.-P. MOCQUARD, 1969. Contribution a l'étude de la physiologie sexuelle femelle de *Ligia oceanica* L. (Crustacé Oniscoïde). Differenciation des oostégites et contrôle neurohumoral de la maturation ovarienne. *C.R. Acad. Sci. Paris*, **269**: 733–736.

BESSÉ, G., AND C. DONADEY, 1972. Contrôle neuro-endocrine du fonctionnement des caecums digestifs des Crustacés Isopodes. Etude chez deux Oniscoides: *Porcellio dilatatus* (Brandt) et *Ligia oceanica* (L.). *C.R. Acad. Sci. Paris*, **275**: 2387–2390.

Bessé, G., and J. Maissiat, 1971. Action de la glande de mue sur la vitellogenèse du Crustacé Isopode *Porcellio dilatatus* (Brandt). *C.R. Acad. Sci. Paris,* 273: 1975–1978.

Blanchet, M. F., P. Porcheron, and F. Dray, 1979. Variations du taux des ecdysteroïdes au cours des cycles de mue et de vitellogenèse chez le Crustacé Amphipode, *Orchestia gammarellus. Int. J. Invert. Reprod.* **1**: 133–139.

Bliss, D. E., 1966. Relation between reproduction and growth in decapod crustaceans. *Am. Zool.,* **6**: 231–233.

Charmantier, G., M. Ollé, and J.-P. Trilles, 1976. Aspects du dosage de l'ecdystérone chez *Sphaeroma serratum* (Crustacea, Isopoda, Flabellifera) et premiers résultats. *C.R. Acad. Sci. Paris,* **283**: 1329–1331.

Charmantier, G., and J.-P. Trilles, 1975. Aspects du controle nerveux des phenomènes de la mue chez *Sphaeroma serratum* (Fabricius, 1787) (Crustacea, Isopoda, Flabellifera). *C.R. Acad. Sci. Paris,* **280**: 2231–2234.

Charniaux-Cotton, H., 1973. Description et contrôle de l'ovogenèse chez les Crustacés superieurs. *Inst. Nat. Rech. Agron. Ann. Biol.* **13**: 21–30.

Charniaux-Cotton, H., and L. H. Kleinholz, 1964. Hormones in invertebrates other than insects. Pages 135–198 in G. Pincus, K. V. Thimann, and E. V. Astwood, Eds., *The Hormones,* Vol. 4, Academic Press, N. Y.

Delaleu, J. C., and A. Holley, 1976. Contribution of an electrogenic pump to the resting membrane polarization in a crustacean heart. *J. Exp. Biol.* **64**: 59–74.

Drach, P., 1939. Mue et cycle d'intermue chez les Crustacés Décapodes. *Ann. Inst. Océanog. (Monaco),* **19**: 103–391.

Drach, P., and C. Tchernigovtzeff, 1967. Sur la méthode de détermination des stades d'intermue et son application générale aux Crustacés. *Vie Milieu,* **18**: 595–609.

Gabe, M., 1966. *Neurosecretion,* Pergamon, Oxford, 872 pp.

Heeley, W., 1941. Observations on the life-histories of some terrestrial isopods. *Proc. Zool. Soc. Lond. B.,* **111**: 79–149.

Hoarau, F., and M. Hirn, 1978. Evolution de taux des ecdystéroïdes au cours du cycle de mue chez *Helleria brevicornis* (Isopode terrestre). *C.R. Acad. Sci. Paris,* **286**: 1443–1446.

Legrand, J.-J., 1955. Rôle endocrinien de l'ovaire dans la differenciation des oostégites chez les Crustacés Isopodes terrestres., *C.R. Acad. Sci. Paris,* **241**: 1083–1085.

Lindqvist, O. V., 1972. Components of water loss in terrestrial isopods. *Physiol. Zool.,* **45**: 316–324.

Mayes, K. R., and D. M. Holdich, 1975. Water exchange between woodlice and moist environments, with particular reference to *Oniscus asellus. Comp. Biochem. Physiol. A,* **51**: 295–300.

McQueen, D. J., 1976. *Porcellio spinicornis* Say (Isopoda) demography. II. A comparison between field and laboratory data. *Can. J. Zool.,* **54**: 825–842.

McQueen, D. J., and C. G. H. Steel, 1980. The role of photoperiod and temperature in the initiation of reproduction in the terrestrial isopod *Oniscus asellus* (Linnaeus), *Can. J. Zool.,* **58**: 235–240.

Merriam, H. G., 1971. Sensitivity of terrestrial isopod populations (*Armadillidium*) to food quality differences. *Can. J. Zool.,* **49**: 667–674.

Meusy, J.-J., M.-F. Blanchet, and H. Junera, 1977. Mue et vitellogenèse chez le Crustacé Amphipode *Orchestia gammarella* Pallas. II. Etude de la synthèse de la vitellogénine ("fraction protéique femelle" de l'hemolymphe) après destruction des organes Y. *Gen. Comp. Endocrinol.,* **33**: 35–40.

Meusy, J.-J., H. Junera, and Y. Croisille, 1974. Données sur la synthese de la fraction protéique femelle chez *Orchestia gammarella* Pallas (Crustacé Amphipode), au cours de l'intermue et chez les femelles en repos sexuel. *C.R. Acad. Sci. Paris,* **279**: 587–590.

Mocquard, J.-P., G. Bessé, P. Juchault, J.-J. Legrand, J. Maissiat, and G. Noulin, 1971. Contribution à l'analyse du contrôle neurohumoral de la croissance, de la mue et de la physiologie sexuelle mâle et femelle chez l'Oniscoïde *Ligia oceanica* L. (Crustacé, Isopode). *Ann. Embryol. Morph.,* **4**: 45–63.

Nemec, B., 1896. Studien über Isopodem. II., *S. B. Bohm. Ges. Wiss.,* 1–55.

Panouse, J. B., 1947. La glande du sinus et la maturation des produits génitaux chez les crevettes. *Bull. Biol. Fr. Belg.* (Suppl.), **33**: 160–163.

Passano, L. M., 1960. Molting and its control. Pages 473–536 in T. H. Waterman, Ed., *The Physiology of Crustacea,* Vol. 1, Academic Press, N. Y.

REAKA, M. L., 1975. Molting in stomatopod crustaceans: I. Stages of the molt cycle, setagenesis, and morphology. *J. Morphol.* **146**: 55–80.

REIDENBACH, J.-M., 1965. Effets d l'ablation du complexe neurosécréteur céphalique chez les femelles du Crustacé Isopode marin *Idotea balthica basteri* Andouin. Premiers résultats. *C.R. Acad. Sci. Paris*, **261**: 4237–4239.

SOCHASKY, J. B., 1973. *Failure to accelerate molting following eyestalk ablation in decapod crustaceans.* Fisheries Research Board of Canada. Technical Report No. 431. 127 pp.

STANDEN, V., 1970. The life history of *Trichoniscus pusillus pusillus* (Crustacea: Isopoda). *J. Zool.*, **161**: 461–470.

SUTTON, S. L., 1972. *Woodlice,* Ginn and Co., London, 144 pp.

TCHERNIGOVTZEFF, C., AND J. RAGAGE-WILLIGENS, 1968. Détermination des stades d'intermue chez *Sphaeroma serratum* (Isopode Flabellifere). *Arch. Zool. Exp. Gén.*, **109**: 305–318.

VRANCKX, R., AND M. DURLIAT, 1978. Comparison of the gradient of setal development of uropods and of scaphognathites in *Astacus leptodactylus*. *Biol. Bull.*, **155**: 627–639.

Reference: *Biol. Bull.*, **159**: 219–230. (August, 1980)

STUDIES ON REPRODUCTION IN THE COMPOUND ASCIDIAN, *SYMPLEGMA REPTANS:* RELATIONSHIP BETWEEN NEURAL COMPLEX AND REPRODUCTION[1]

KEIJI SUGIMOTO AND HIROSHI WATANABE[2]

Zoological Institute, Faculty of Science, Tokyo Kyoiku University, Otsuka, Tokyo, Japan

The neural gland in ascidians was first clearly indicated by Hancock (1868). Many morphological or physiological studies have been made on this structure since then. Earlier investigators attributed various functions to the gland: It was postulated to function as a lymphatic organ (Herdman, 1883), an excretory system (Pérès, 1943; Millar, 1953) or a mucus gland (Roule, 1884). In addition, the possibility that it acts as an endocrine organ has been extensively discussed (Butcher, 1930; Carlisle, 1951; Dodd, 1955) since Julin (1881) first postulated a homology of the neural gland of ascidians with the hypophysis of vertebrates on the basis of its position and origin. However, these studies have resulted in much disagreement, and the function and structure of the gland in ascidians remain controversial. Neurosecretory cells have been shown to occur in the neural ganglion of ascidians, one of the main components of the neural complex in this animal (Dawson and Hisaw, 1964; Lane, 1972). Great interest concerns whether the ascidian ganglion has a gonadotropic function (Hisaw *et al.*, 1966; Sengel and Georges, 1966; Bouchard-Madrelle, 1967). However, an acceptable interpretation of function(s) of the neural ganglion and gland has not been proposed.

Many recent studies on the neurosecretory phenomena in various invertebrates other than ascidians have been carried out (Tombes, 1970; Golding 1974, for reviews). Accumulating evidence from such studies indicates that the neurosecretory system in invertebrates plays an important role in reproductive activity. The neurosecretory system may serve a similar function in ascidians. The present study is focused primarily on the gonadotropic function of the neural complex in a compound ascidian, *Symplegma reptans.* As an approach to this subject, the functional criteria for neurosecretory status suggested by Bern (1962) were applied and a possible role for the neural complex in reproductive activity in this species is postulated.

MATERIALS AND METHODS

Symplegma reptans Oka, a compound ascidian, was used in this study. Colony fragments were attached to glass plates and reared in the bay at the Shimoda Marine Biological Station (presently Shimoda Marine Research Center), Shimoda, Japan.

Zooids with few ova were used for the ganglion-ablation experiment, since gonads of older zooids with several larvae have a tendency to degenerate after isolation from the colony. Operations were done on solitary zooids to avoid possible influences from unoperated zooids in a colony. Zooids were made solitary on the glass plates by removing other zooids and, thereafter, newly formed buds. A small incision was made along the neural complex through the tunic and the ganglion was cut off with a tungsten wire with its tip bent in a hook. Control zooids received only the incision. To confirm completeness of ganglion removal, physiological response of the operated zooids to exogenous stimuli was examined by touching a needle to the buccal aperture, which does not contract quickly in a zooid having no ganglion. Histological examinations were also made. The operated zooids were hung upside-down so that their excretions could easily pass through an atrial siphon.

---

[1] Contribution No. 360 from the Shimoda Marine Research Center.

[2] Present addresses: Sugimoto: Department of Anatomy, Nippon Medical School, 1–1 Sendagi, Bunkyo-ku, Tokyo 113, Janpan; Watanabe: Shimoda Marine Research Center, University of Tsukuba, Shimoda 5–10–1, Shizuoka-ken 415, Japan.

Electrical stimulation of the ganglion was carried out to release neurosecretory materials from neurosecretory axon-terminals into the circulatory system. After surgical exposure of the ganglion as described above, electrical shocks were administered to the ganglion, which was sucked into a glass needle containing the stimulatory electrode (platinum wire, 100 μ in diameter). Rectangular pulses of 10–150 V amplitude and 1–5 msec duration were applied at the rate of 2–50 shocks per sec for 15–30 min. Controls received only the operation without the electrical shocks. Effects of the stimulation on the neurosecretory cells in the ganglion were examined histologically by applying Gomori's paraldehyde-fuchsin (Gomori's stain) to the fixed materials within 1 hr after stimulation. Small colonies composed of six to seven sexually mature zooids and one to two sexually immature zooids were used in bioassays to examine the physiological effect of the stimulation on the growth of oocytes. Electrical stimulation was done on mature zooids and the growth rate of oocytes in immature zooids was recorded.

For histological study, materials were fixed in 2.5% phosphate-buffered glutaraldehyde at pH 7.4 for 2.5 hr, followed by 1% phosphate-buffered osmium tetroxide at pH 7.4 for 1 hr. Materials then were dehydrated in ethanol series and embedded in Epon. To calculate numbers of neurosecretory cells in the ganglion, sections were prepared every 3 μ on a Porter Blum ultramicrotome and stained with Gomori's stain after removal of resin according to the method of Imai *et al.* (1968). Thin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HS-9 electron microscope at 50 kV.

RESULTS

Structure of the neural complex in Symplegma

The components of the neural complex in *Symplegma* are the same as those in other ascidians: ganglion, neural gland, and hypophysial duct. The cells of the neural gland have a long axis of about 12 μ and range from cuboidal to irregular in contour. The glandular cells often appear to be vacuolated and were not specificially stained by Gomori's stain. Electron micrographs of the cells were characterized by a number of large cytoplasmic vacuoles filled with fibrous materials. Structural connections between the neural gland and the ganglion were not observed. The cells of the hypophysial duct did not stain at all with Gomori's stain.

The ganglionic cells are relatively small, measuring about 6–7 μ in long axis, and surround a central core of nerve fibers. Some of them were positively stained by Gomori's stain, as has been shown in other ascidians. Furthermore, they could be classified ultrastructurally into three types by cytoplasmic contents: 1) Cells having abundant mitochondria and no granular components. 2) Cells having numerous electron-dense granules 100–140 mμ in diameter (Fig. 1-A). 3) Cells having electron-transparent granules 240–300 mμ in diameter (Fig. 1-B). The

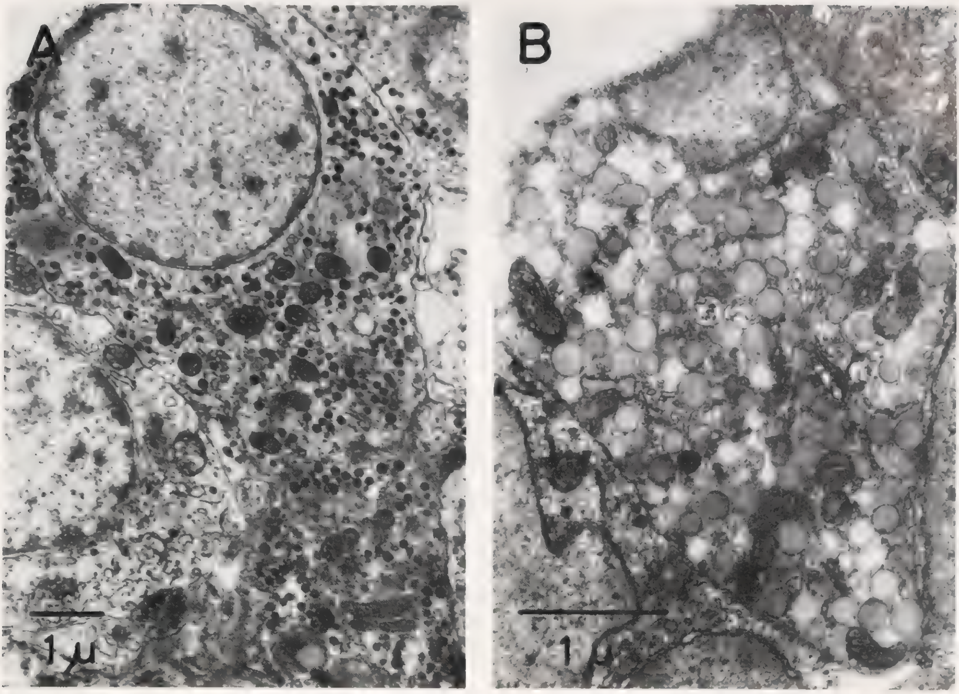


FIGURE 1. Two types of neurosecretory cells in the ganglion: (A) neurosecretory cells containing electron-dense small granules with diameters of 100–140 mμ. (B) a neurosecretory cell containing electron-transparent granules with diameters of 240–300 mμ.

latter two cell types have features characteristic of neurosecretory cells described in many other animals. The last cell type could not be distinguished histochemically from the second cell type and was rarely observed in electron micrographs. Almost all neurosecretory cells in the ganglion belong to the second cell type.

Axon bundles containing the same types of granules as in nuerosecretory cells were observed often only near the ganglion. The bundles were located just beneath the epidermis (Fig. 2). Other bundles of unmyelinated nerve fibers ran deeper and contained no granular components. The axon terminal of each neurosecretory cell or neurohemal organ, where a number of neurosecretory cells' axons terminated, was not found in the present study.

Relationship between the neurosecretory cell and gonad development

During early gonad development, ganglionic cells showed little cytoplasmic specialization for producing granules. Only a few unidentified granules were found near the poorly developed Golgi apparatus. Gomori-postive-staining cells were not found in the ganglion at this stage. When oocytes attained 50 μ in diameter, still in the stage of previtellogenesis, some ganglionic cells produced cytoplasmic granules and reacted positively with Gomori's stain. Such cells increased in number with oocyte development. Their rapid increase was particularly noticeable in the period when the eldest oocyte grew from the stage of previtellogenesis to vitellogenesis. After this stage, the number of cells remained constant (Fig. 3).

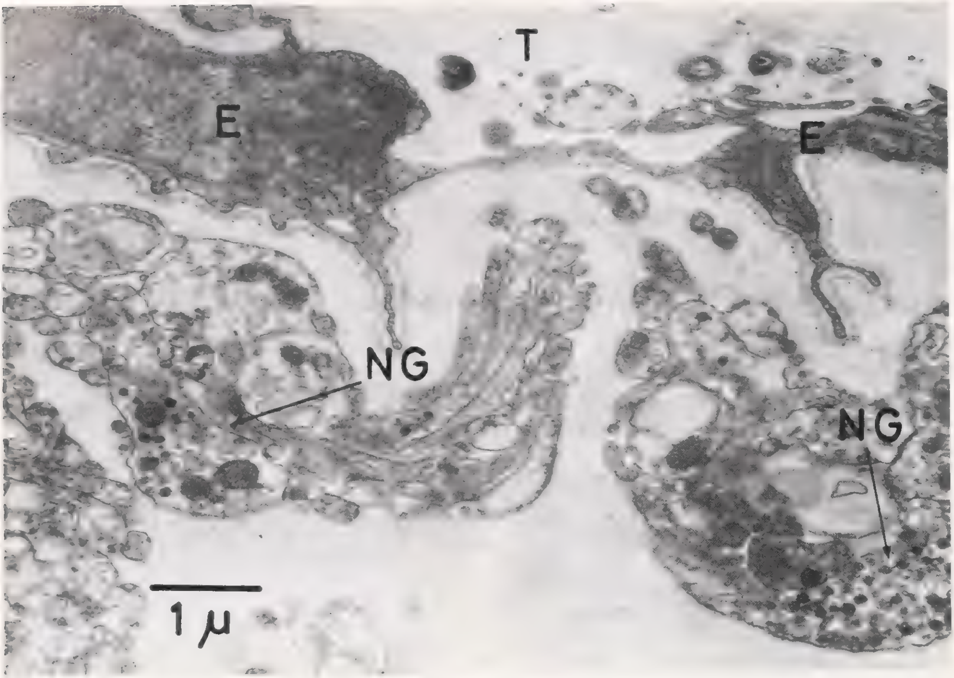


FIGURE 2. Axons distributed near the ganglion. Note the axons containing neurosecretory granules. E = epidermis; NG = neurosecretory granule; T = tunic.

The relationship between the neurosecretory cells and gonad development was also investigated under conditions disadvantageous for growth of oocytes. First, the number of Gomori-positive cells was examined in young solitary zooids. Zooids having small oocytes, 30 μ in diameter, were isolated and stripped of buds. During the 15 days after isolation, their oocytes grew little and remained previtellogenic, 30–50 μ in diameter. Oocytes of same-aged zooids in the colony that served as control attained 100–150 μ in diameter during the same period. The number of Gomori-positive cells in the experimental zooids averaged 35.5 ± 8.9 cells per ganglion. That of control zooids was 112.0 ± 13.9 cells per ganglion (Fig. 4), the same as in isolated, sexually mature zooids with several larvae in their brood sacs. Although the experimental zooids continued growth and bud formation, their gonads degenerated completely by 7 days after isolation. The number of Gomori-positive cells decreased linearly and reached about half that of the control zooids in 10 days (Fig. 4). With electron microscopy, many lysosomes were observed in the neurosecretory cells of ganglia at this stage, and a number of specific granules were contained in such structures (Fig. 5).

Effects of ganglion ablation on gonad development

The growth rate of zooids decreased slightly by 3–5 days after ganglion ablation, but thereafter increased in both control and experimental groups (Fig. 6). Here, small oocytes less than 70 μ in diameter and in the stage of previtellogenesis are called Stage 1, and large vitellogenic oocytes 70–200 μ in diameter are called Stage 2. Stage 1 oocytes decreased to as few as 75% of the initial number, but their complete disintegration was not observed (Fig. 7-A). On the other hand, Stage 2 oocytes were greatly damaged by the operation and their number decreased to less than half within one day and approached zero by 10 days after operation (Fig. 7-B). Neither fully grown ova, which were more than 200 μ in diameter, nor testes were affected by ganglion ablation. The ova in operated zooids ovulated normally into the brood sacs.

Effects of electrical stimulation of the ganglion on gonad development

Attempts to transplant ganglia into a ganglion-ablated zooid were unsuccessful. In place of transplanting experiments, electrical stimulation of the ganglion was carried out. Rectangular pulses of 40 V amplitude and 5 msec duration were applied at 10–20 shocks per sec for 15 min. Under these conditions, the Gomori-positive cells in the ganglion decreased in number to about 50% of control. The number of cells in the stimulated zooids averaged 56.7 ± 23.1 cells per ganglion and that of control zooids averaged 110.8 ± 15.0. The possibility that electrical stimulation to the ganglion accelerated release of granules from the neurosecretory cells led us to examine the effects of ganglion stimulation on gonad development. Six to seven sexually mature zooids were stimulated electrically as described above. If oocytes of the immature zooids were still in the stage of previtellogenesis, growth of such oocytes was accelerated by the stimulation (Fig. 8-A). On the eighth day after stimulation, oocytes in the unstimulated controls averaged 111.4 μ in diameter, while oocytes in the stimulated group averaged 142.3 μ in diameter. This difference was statistically significant. On the other hand, if oocytes of the

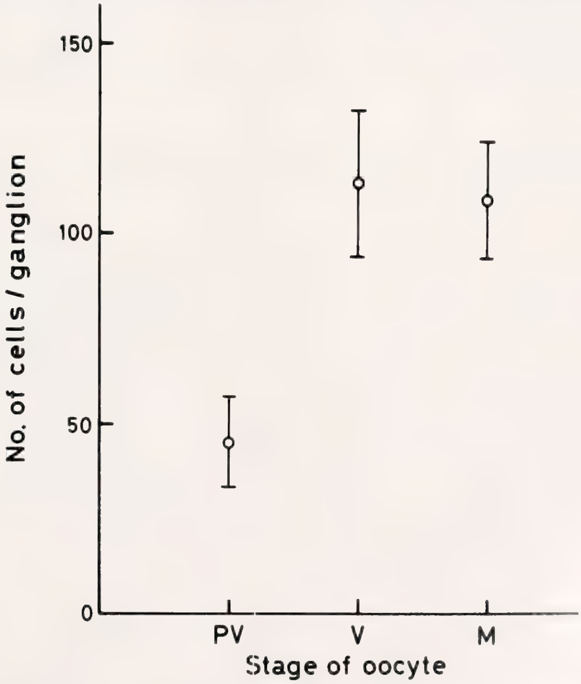


FIGURE 3. Variation in the number of Gomori-positive-cells in the ganglion at different stages of gonad development. Means and ranges of estimated cell numbers per ganglion in 13 zooids at each developmental stage are depicted. PV = previtellogenic; V = vitellogenic; M = fully grown ova.

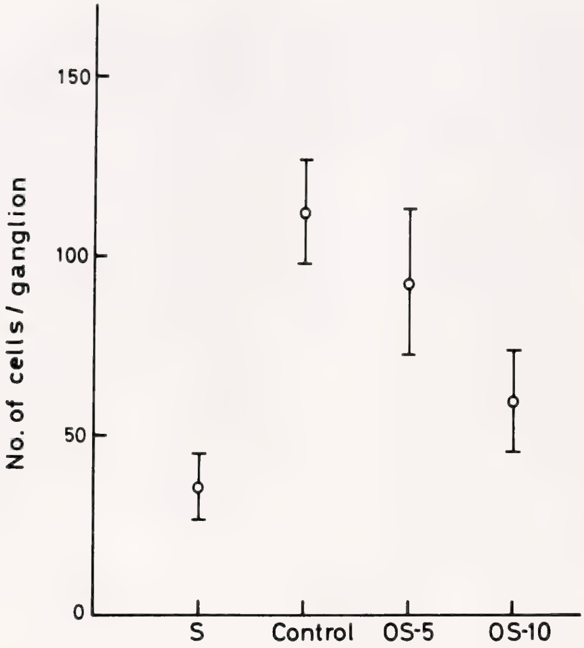


FIGURE 4. Variation in number of Gomori-positive cells under conditions disadvantageous for gonad development. Ten young zooids with previtellogenic oocytes and 10 old zooids with several larvae were isolated from the colony and reared alone for 15 and 10 days, respectively. Thirteen zooids with vitellogenic oocytes served as controls. Means and ranges of estimated cell numbers per ganglion in each zooid are depicted. S = young solitary zooid; OS-5 = 5-day-old solitary zooid after isolation; OS-10 = 10-day-old solitary zooid after isolation.

immature zooids were vitellogenic, growth of these oocytes was almost the same in both the stimulated and control groups (Fig. 8-B).

DISCUSSION

Although many morphological and physiological studies on the neural gland of ascidians have been made, the functions of this structure have not yet been fully resolved. No intimate structural relationship between neural gland and neural ganglion has been observed in *Symplegma*. Moreover, cells with membrane-bound secretory granules, which characterize the secretory cells of vertebrate adenohypophysis, have not been found in the neural gland. Instead of such granules, a number of vacuoles are found in the glandular cells, as reported by Lane (1971). The fact that degenerating cells show a high degree of cytoplasmic vacuolation suggests that the glandular cells may secrete their products by holocrine secretion, as do sebaceous or meibomian glands in vertebrates. Histological characteristics reveal that the glandular cells are distinctly different from typical neurosecretory cells in many species. In connection with secretory cells, it is interesting that neurosecretory cells occur in the neural ganglia of ascidians (Dawson and Hisaw, 1964; Chambost, 1966; Lane, 1972), which lies immediately adjacent to the neural gland. In *Symplegma*, two types of neurosecretory cells can be distinguished by electron microscopy. The character of granules surely is a reflection of function, but whether these cells are concerned with different functions or not must be discerned in the future.

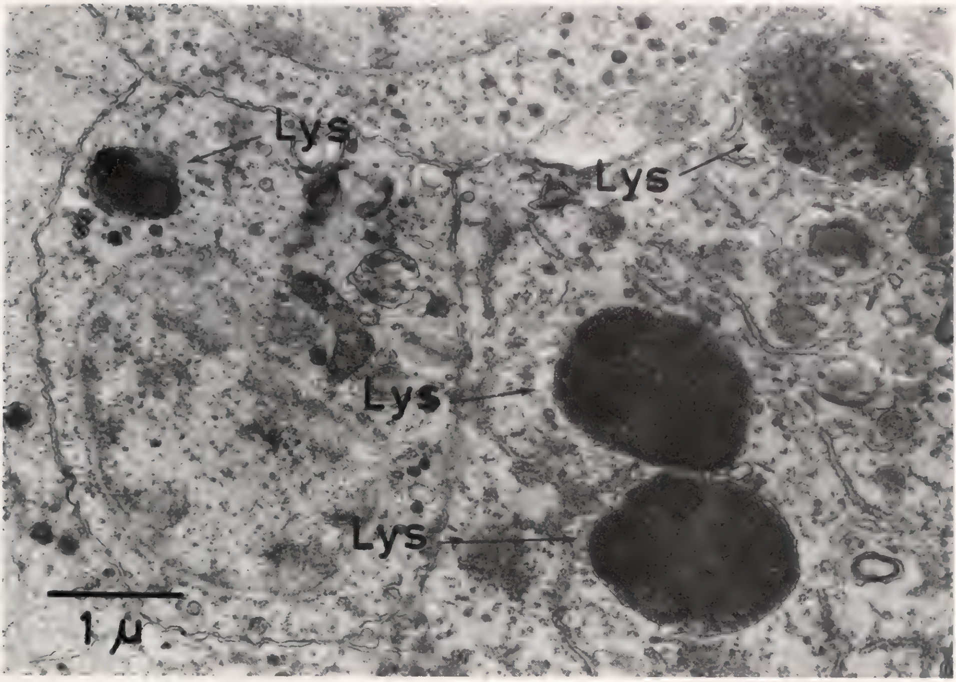


FIGURE 5. Secondary lysosomes appearing in the neurosecretory cells of 10-day-old solitary zooids after isolation. Lys = secondary lysosome.

A neurohemal region where neurosecretory materials are released into the circulatory system could not be identified in the present study. Axonal endings of neurosecretory cells may exist near the ganglion, since axons containing the neurosecretory granules were found only near the ganglion. A well-defined neurohemal organ such as the median eminence of vertebrates, the sinus gland of crustaceans, and the corpus cardiacum of insects was not found in *Symplegma*. Such diffuse and short-range distribution of the axon terminals is known in the ganglia of molluscs (Frazier *et al.*, 1967; Toevs and Brackenburry, 1969; Loh *et al.*, 1973).

Many studies concerning the mechanism of reproduction in ascidians have been carried out since Julin (1881) postulated a homology of the neural gland with the hypophysis of vertebrates. Previous studies examining possible gonadotropic potency of the neural complex showed contradictory results: Butcher, 1930; Hogg, 1937; and Carlisle, 1951, concluded that there was a positive function but Dodd, 1955, and Sawyer, 1959, showed negative function. However, the bioassay system of earlier investigators, using tissues of vertebrates, has to be reconsidered because there is no reason to suppose that similar phenomena in such phylogenetically distinct organisms are regulated by the same materials. For example, gonadotropin and progesterone affect oocyte maturation in vertebrates, but the same phenomenon in starfish is regulated by a peptide hormone from the nervous system and 1-methyladenine (see Kanatani, 1973, for review). Therefore, in examining hormonal activity of a species, animals of at the very least the same phylum must be used in order for bioassays to have relevance.

Many studies concerning the function of neurosecretory cells on gonad development indicate that distinctly contrasting control mechanisms operate in various

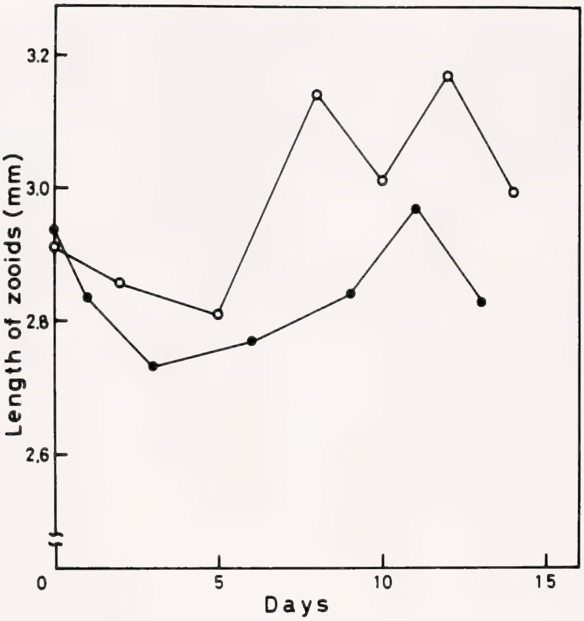


FIGURE 6. Effects of ganglion ablation on growth of zooids. Average growth curves of 35 operated zooids (solid circles) and 27 control zooids (open circles) are depicted.

species. For example, control of sexual reproduction by the neurosecretory system in some species is inhibitory in character, whereas a positive gonadotropic influence exists in other species. Neurosecretory materials in *Hydra* act somewhat like a growth hormone and inhibit gonad development (Schaller, 1973; Schaller and Gierer, 1973). Clearly, neurosecretory cells in *Hydra* affect asexual reproduc-

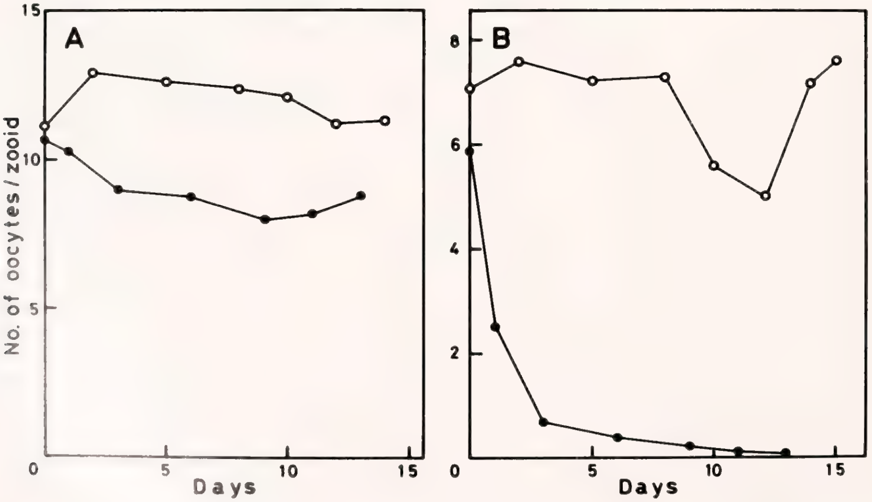


FIGURE 7. Effects of ganglion ablation on oocyte growth. Average oocyte number in 35 operated zooids (solid circles) and 27 control zooids (open circles) are depicted: (A) variation in the number of oocytes in the stage of previtellogenesis; (B) variation in the number of vitellogenic oocytes.

tion, that is, zooid growth and bud formation. The same inhibitory influences of neurosecretory cells were shown in nemertines (Bierne, 1970) and in nereid polychaetes (Clark, 1965; Baskin and Golding, 1970). On the other hand, a positive function has been demonstrated in many other invertebrates, such as turbellarians (Grasso and Quaglia, 1971), molluscs (Geraerts and Algera, 1976; Wijdenes and Runham, 1976), non-nereid polychaetes (Howie, 1966; Gouedard-Couadou and Vicente, 1971), and arthropods (Tombes, 1970; Adiyodi and Adiyodi, 1970 for reviews). In *Symplegma*, the pattern of occurrence of Gomori-positive neurosecretory cells is different from that of *Hydra*. Active zooid growth and bud formation were observed in both young and aged zooids isolated from the central region of a colony, but they showed only a few such neurosecretory cells in their ganglia. As shown in Figure 3, variation in the number of Gomori-positive neurosecretory cells is superficially related to oocyte development. That is, the cell number increases rapidly when the oocyte proceeds from previtellogenesis to vitellogenesis.

In addition, these cells decrease in number under conditions disadvantageous for gonad development. Thus, the neurosecretory cells in *Symplegma* seem to be functionally insignificant in asexual reproduction but to have some significance in sexual reproduction. If this is so, surgical removal of the ganglion should affect gonad development. The most distinctive effect of the operation was the breakdown of oocytes in Stage 2, while young Stage 1 oocytes were mildly affected by the operation. Similar results have been obtained in *Ciona* (Bouchard-Madrelle, 1967). But studies in *Chelyosoma* showed that neural-complex ablation has no effect on gonad development (Hisaw *et al.*, 1966). Additional studies to compare single ascidians with compound ones or to compare species with distinct

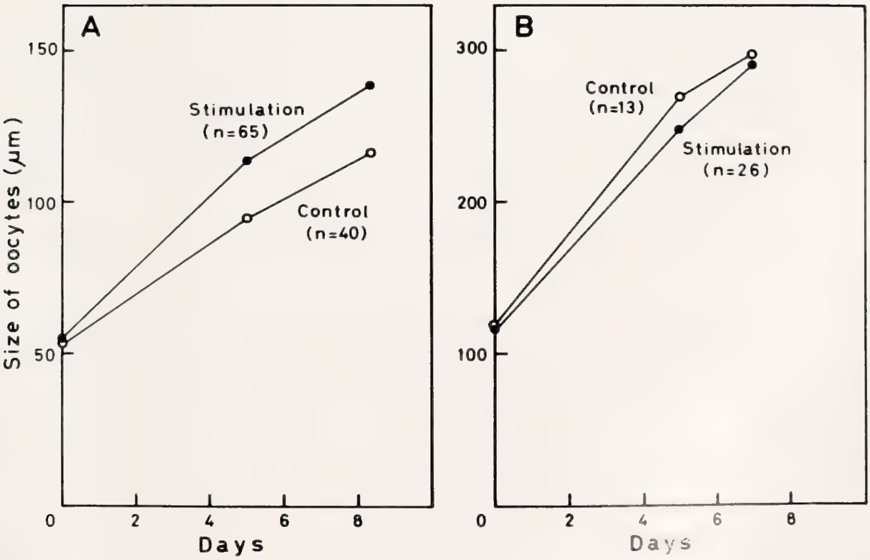


FIGURE 8. Effects of electrical stimulation of the ganglion on the growth of oocytes. Average growth curves of oocytes in the stimulated and control groups are depicted. Figures in parentheses show the number of oocytes used in each group: (A) growth of oocytes in the stage of previtellogenesis; (B) growth of vitellogenic oocytes.

breeding seasons with those without limited breeding seasons will be needed to elucidate the differences between them.

As shown in Sugimoto and Watanabe (1980) delay of oocyte development in zooids reared from their early developmental stages in isolation may result not from the slow rate of synthesis of vitelline, but rather from prolongation of the previtellogenic period. This finding and the results in the present study suggest that initiation of vitellogenesis may be the critical point in oocyte maturation. If we accept this view, the effect of electrical stimulation on the growth of young oocytes in the stage of previtellogenesis can be interpreted as indicating that the stimulation speeds the initiation of vitellogenesis. Similar effects of electrical stimulation on gonad development have been shown in insects (Highnam, 1962, Wigglesworth, 1964).

Thus, the ganglion in this species may have an important role in the process of vitellogenesis. To substantiate this possibility, it is necessary to examine the dynamics of neurosecretory materials at each stage of gonad development, using an *in vitro* system to see whether or not neurosecretory materials act directly on the gonad.

We are grateful to Mr. Masahiko Sato, Dartmouth College, for his helpful suggestions and a critical reading of the manuscript. We wish to acknowledge our indebtedness to the staff of the Shimoda Marine Research Center for their kindness in providing many conveniences throughout the course of the study. This study was supported, in part, by a grant-in-aid for Scientific Research from the Ministry of Education of Japan.

SUMMARY

1. The neural complex was examined for induction of sexuality in a compound ascidian, *Symplegma reptans*. The number of Gomori-positive neurosecretory cells in the neural ganglion increases rapidly while oocyte development proceeds from previtellogenesis to vitellogenesis and is constant in sexually mature zooids. However, such cells decrease in number in conditions disadvantageous for gonad development. The decrease in cell numbers in isolated aged zooids may result from lysosomal digestion of granules.

2. Fully grown ova and previtellogenic oocytes suffer mild influences from ganglion ablation. However, vitellogenic oocytes always suffer profound damage from the operation.

3. In sexually mature zooids, electrical stimulation to the neural ganglion leads to decrease in the number of Gomori-positive neurosecretory cells. The stimulation accelerates growth of previtellogenic oocytes, but does not affect oocytes in other developmental stages.

4. These findings suggest that the ganglionic neuroscretory cells in *Symplegma* may function in sexual reproduction, especially in the vitellogenesis.

LITERATURE CITED

Adiyodi, K. G., and R. G. Adiyodi, 1970. Endocrine control of reproduction in decapod Crustacea. *Biol. Rev.*, **45**: 121–165.

Baskin, D. G., and D. W. Golding, 1970. Experimental studies on the endocrinology and reproductive biology of the viviparous polychaete annelid, *Nereis limnicola* Johnson. *Biol. Bull.*, **139**: 461–475.

Bern, H. A., 1962. The properties of neurosecretory cells. *Gen. Comp. Endocr., Suppl. I:* 117–132.

Bierne, J., 1970. Recherches sur la différenciation sexuelle au cours de l'ontogenése et de la régénération chez le némertien *Lineus ruber* (Müller). *Ann. Sci. Nat. Zool. Biol. Anim.* **12**: 181–198.

Bouchard-Madrelle, C., 1967. Influence de l'ablation d'une partie ou de la totalié de complexe neural sur le fonctionnement des gonades de *Ciona intestinalis* (Tunicier, Ascidiacé). *C.R. Acad. Sci. Ser. D.*, **264**: 2055–2059.

Butcher, E. O., 1930. The pituitary in the ascidians (*Molgula manhattensis*). *J. Exp. Zool.*, **57**: 1–11.

Carlisle, D. E., 1951. On the hormonal and neural control of the release of gametes in ascidians. *J. Exp. Biol.*, **28**: 463–472.

Chambost, D., 1966. Le complexe neural de *Ciona intestinalis* L. (Tunicier, Ascidiacea). Étude comparative du ganglion nerveux et de la glande asymétrique aux microscopes optique et électronique. *C.R. Acad. Sci. Ser. D.*, **263**: 969–971.

Clark, R. B., 1965. Endocrinology and the reproductive biology of the polychaetes. *Oceanog. Marine Biol. Ann. Rev.*, **3**: 211–255.

Dawson, A. B., and F. L. Hisaw, Jr., 1964. The occurrence of neurosecretory cells in the neural ganglion of tunicates. *J. Morphol.*, **114**: 411–423.

Dodd, J. M., 1955. The hormones of sex and reproduction and their effects in fish and lower chordates. Pp. 166–187 in I. C. Jones and P. Eckstein, Eds., *Comparative Physiology of Reproduction, Memoirs of the Society for Endocrinology.* Cambridge Univ. Press, London.

Frazier, W. T., E. R. Kandel, I. Kupfermann, R. Waziri, and R. E. Coggeshall, 1967. Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica.* *J. Neurophysiol.*, **30**: 1288–1351.

Geraerts, W. P. M., and L. H. Algera, 1976. The stimulating effect of the dorsal-body hormone on cell differentiation in the female accessory sex organs of the hermaphrodite freshwater snail, *Lymnaea stagnalis, Gen. Comp. Endocrinol.*, **29**. 109–118.

Golding, D. W., 1974. A survey of neuroendocrine phenomena in non-arthropod invertebrates. *Biol. Rev.*, **49**: 161–224.

Gouedard-Couadou, E., and N. Vicente, 1971. Sur les corrélations neuroendocrines chez *Spirobis* (*Pileolaria*) *militaris* (Claparéde), 1870 (Polychaeta, Serpulidae). *C.R. Séances Soc. Biol. Fil.*, **165**: 1092–1096.

Grasso, M., and A. Quaglia, 1971. Studies on neurosecretion in planarians. III. Neurosecretory fibers near the testis and ovaries of *Polycelis nigria.* *J. Submicrosc. Cytol.*, **3**: 171–180.

Hancock, A., 1868. On the anatomy and physiology of the Tunicata. *J. Linn. Soc. Lond. Zool.*, **9**: 309–346.

Herdman, W. A., 1883. The hypophysis cerebri in Tunicata and Vertebrata. *Nature*, **28**: 284–286.

Highnam, K. C., 1962. Neurosecretory control of ovarian development in *Schistocerca gregaria.* *Q. J. Microsc. Sci.*, **103**: 57–72.

Hisaw, F. L., Jr., C. R. Botticelli, and F. L. Hisaw, 1966. A study of the relation of the neural gland-ganglion complex to gonadal development in an ascidian, *Chelyosoma productum* Simpson. *Gen. Comp. Endocrinol.*, **7**: 1–9.

Hogg, B. M., 1937. Subneural gland of ascidian (*Polycarpa tecta*): An ovarian stimulating action in immature mice. *Proc. Soc. Exptl. Biol. Med.*, **35**: 616–618.

Howie, D. I. D., 1966. Further data relating to the maturation hormone and its site of secretion in *Arenicola marina* Linnaeus. *Gen. Comp. Endocrinol.*, **6**: 347–361.

Imai, Y., A. Sue, and A. Yamaguchi, 1968. A removing method of the resin from Epoxy-embedded sections for light microscopy. *J. Electron Microsc.*, **17**: 84–85.

Julin, C., 1881. Recherches sur l'organization des Ascidies simples. Sur l'hypophyse et quelques organes qui s'y rattachent, dans les genres *Corella, Phallusia,* et *Ascidia.* *Arch. Biol.*, **2**: 59–126.

Kanatani, H., 1973. Maturation-inducing substance in starfishes.. *Int. Rev. Cytol.*, **35**: 253–298.

Lane, N. J., 1971. The neural gland in tunicates: Fine structure and intracellular distribution of phosphatases. *Z. Zellforsch.*, **120**: 80–93.

Lane, N. J., 1972. Neurosecretory cells in the cerebral ganglion of adult tunicates: Fine structure and distribution of phosphatases. *J. Ultrastruct. Res.*, **40**: 480–497.

LOH, Y. P., Y. SARNE, AND H. GEINER, 1973. Heterogeneity of proteins synthesized, stored, and released by bag cells of *Aplysia californica*. *J. Comp. Physiol.*, **100**: 283–295.

MILLAR, R. H., 1953. Ciona. Pp. 1–123 in L. S. Colman, Ed., *Liverpool Mar. Biol. Comm. Mem.*, Vol. 35. Univ. Press, Liverpool.

PÉRÈS, J. M., 1943. Recherches sur le sang et les organes neuraux des Tuniciers. *Ann. Inst. Oceanogr. Monaco*, **21**: 229–359.

ROULE, L., 1884. Recherches sur les Ascidies simples des cotés de Provence (Phallusiadées). *Ann. Mus. Marseille*, **2**: 1–270.

SAWYER, W. H., 1959. Oxytocic activity in the neural complex of two ascidians, *Chelyosoma productum* and *Pyura haustor*. *Endocrinology*, **65**: 520–523.

SCHALLER, H. C., 1973. Isolation and characterization of a low-molecular weight substance activating head and bud formation in *Hydra*. *J. Embryol. Exp. Morphol.*, **29**: 27–38.

SCHALLER, H. C., AND A. GIERER, 1973. Distribution of head-activating substance in *Hydra* and its location in membranous particles in nerve cells. *J. Embryol. Exp. Morphol.*, **29**: 39–52.

SENGEL, P., AND D. GEORGES, 1966. Effects de l'éclairement et de l'ablation du complexe neural sur la pont de *Ciona intestinalis* L. (Tunicier, Ascidiacé). *C. R. Acad. Sci. Ser. D.*, **263**: 1876–1879.

TOEVS, L., AND R. BRACKENBURRY, 1969. Bag cell-specific proteins and the humoral control of egg-laying in *Aplysia californica*. *Comp. Biochem. Physiol.*, **29**: 207–216.

TOMBES, A. S., 1970. *An Introduction to Invertebrate Endocrinology*. Academic Press, New York. 203 pp.

WIGGLESWORTH, V. B., 1964. The hormonal regulation of growth and reproduction in insects. *Adv. Insect Physiol.*, **2**: 247–336.

WIJDENES, J., AND N. W. RUNHAM, 1976. Studies on the function of the dorsal bodies of *Agriolimax reticulatus* (Mollusca; Pulmonata). *Gen. Comp. Endocrinol.*, **29**: 545–551.

Reference: *Biol. Bull.*, 159: 231–246. (August, 1980)

THE ZOOGEOGRAPHY AND DIETARY INDUCTION OF BIOLUMINESCENCE IN THE MIDSHIPMAN FISH, *PORICHTHYS NOTATUS*

JON A. WARNER[1] AND JAMES F. CASE

Department of Biological Sciences, University of California, Santa Barbara, California 93106

The light generating systems of *Porichthys notatus,* the midshipman fish, cross react with those of the ostracod *Vargula* (= *Cypridina*) *hilgendorfii* (Cormier *et al.,* 1967), indicating that *Porichthys* might be able to utilize exogenous luciferin to support luminescence. This remarkable possibility is not wholly unexpected since some fishes in the two genera *Apogon* and *Parapriacanthus* are thought to acquire their luciferin from a sympatric ostracod, *V. hilgendorfii* (Haneda *et al.,* 1966, 1969; Tsuji *et al.,* 1971). The research reported here reinforces possible dependence of the midshipman on an exogenous luciferin.

P. notatus occurs in coastal waters from Baja California to Southeastern Alaskan waters (Wilimovski, 1954), a range encompassing many kinds of luminescent organisms which might furnish luciferin (Tsuji *et al.,* 1971). However, luminescent ostracods, the most likely dietary source of luciferin, were not known to occur within the range of the midshipman until Kornicker and Baker (1977) described *Vargula tsujii* (Mycopoda; Cyprindinae). The existence of a luminescent ostracod closely related to *V. hilgendorfii* but, unlike it, sympatric with the southern midshipman population, heightens interest in the possibility that *Porichthys* might normally utilize an exogenous source of luciferin.

Midshipman luminescence is generated by an adrenergically triggered, intracellular, luciferase-mediated oxidation of luciferin in photocytes in hundreds of ventral and lateral photophores (Greene, 1899; Greene and Greene, 1924; Nicol, 1957; Baguet and Case, 1971; Baguet, 1975; Anctil, 1977, 1979a). Case and Strause (1979) and Anctil (1979b) proposed that the characteristic cytoplasmic vesicles and microvillous borders of the photocytes are specializations for uptake and storage of luciferin from large body stores identified by Tsuji *et al.,* 1971.

Specimens of *P. notatus* from Puget Sound are not bioluminescent (Strum, 1968) even though their photophores are morphologically and ultrastructurally similar to those of the southern luminescent fish (Strum, 1969a and b; Anctil and Case, 1976), except for possible reduced amounts of flocculent ground substance in cytoplasmic vesicles of nonluminescent specimens from Puget Sound (unpublished observations). Midshipman fish collected off Southern California contain luciferin in all body tissues, with larger concentrations in photophores (Tsuji *et al.,* 1971), while fish from southern Puget Sound are luciferin deficient, both as embryos and adults (Barnes *et al.,* 1973). Induction of luminescence capability in non-luminescent fish has been achieved with intraperitoneal injection of *V. hilgendorfii* luciferin (Tsuji *et al.,* 1972) and by force feeding of whole dried *V. hilgendorfii* (Barnes *et al.,* 1973). Either technique makes luminescence possible after about 4 days. The bioluminescent response to noradrenaline continues to increase for

---

[1] Present address: Marine Biology Research Division, Scripps Institution of Oceanography, La Jolla, California 92093.

TABLE I

Porichthys bioluminescence survey

Location	Date	Depth (m)	Collecting method	Total trawling time (hr)	Number fish	Size range (mm)	Sex ratio (♂/♀)	Fluorescence %/number	Bioluminescence %/number
Baynes Sound, B. C. 49° 30′ N/124° 48′ W	Aug. 77	0–30	intertidal	—	32 10	27–31 juveniles 180–200 adults	 1.00/0	0/32 0/10	0/32 0/10
Imperial Eagle Channel, B. C. 48° 51′ N/125° 15′ W	Aug. 77	100–120	50′ shrimp trawl	—	30	125–265	0.166/0.834	0/30	0/12
Saanich Inlet, B. C. 48° 34′ N/123° 30′ W	Oct. 76	200	—	—	7	181–243		0/7	0/7
Port Orchard, WA* 47° 45′ N/122° 30′ W	1971–1972		—	—	308			0/308	0/308
Yaquina Head, OR 44° 37′ N/124° 25′ W	Sep. 77	30–100	16′ otter trawl	6	0				
Yaquina Head, OR 44° 37′ N/124° 34′ W	Sep. 77	200	74′ shrimp trawl	24	0				
Yaquina Bay, OR 44° 36′ N/124° 00′ W	Sep. 77	2–10	16′ otter trawl	1	0				
Cape Arago, OR 43° 30′ N/124° 35′ W	Sep. 77	200	72′ shrimp trawl	6	0				
San Pablo St., SF Bay, CA 37° 57′ N/122° 26′ W	May 77	10–30	43′ otter trawl	4	65	75–310	0.75/0.25	85/65	85/65
Gulf of the Farallons, CA 37° 08′ N/124° 15′ W	Jun. 77	20–140	43′ otter trawl	4	281	85–300		85/281	
Moss Landing, CA 36° 51′ N/121° 55′ W	Jun. 77	12–100	16′ otter trawl	0.5	31	160–310		100/31	100/31
Pt. Cabrillo, Monterey, CA 36° 38′ N/121° 54′ W	Jun. 77	25	16′ otter trawl	0.25	39	81–198		100/39	100/15
Santa Barbara Channel, CA 34° 22′ N/119° 30′ W	Jun.–Nov. 77	3–20	scuba		83	27–330	0.99/0.01	100/83	100/41
Santa Monica Bay, CA 33° 55′ N/118° 42′ W	Mar. 77	500	15′ balloon trawl	—	12	160–210		100/12	100/1

* See Barnes *et al.*, 1973.

several weeks without further feeding but ultimately disappears after several months. Luminescence is inducible with very low doses of *V. hilgendorfii* luciferin, or feeding with only one whole, dried specimen. Depletion of luciferin stores does not occur after injections of norepinephrine, which produces long-lasting luminescence. Enzymatically- and air-oxidized *V. hilgendorfii* luciferin, synthetic oxyluciferin, and three luciferin analogs failed to induce luminescence in non-luminescent fish (Barnes *et al.,* 1973; Tsuji *et al.,* 1975).

There is a strong correlation between ultraviolet-stimulated photophore fluorescence and ability to luminesce in the midshipman. Photophores of midshipman fish from southern California waters emit green fluorescence when excited with 365 nm u.v. light. Specimens from Hood Canal (Puget Sound) neither luminesce nor fluoresce (Barnes *et al.,* 1973). Fluorescence is also undetectable in larval southern fish before the 28th day of development, while subsequently both fluorescence and luminescence are present (Anctil, 1977). Baguet and Ziets-Nicolas (1979) report that the intensity of photophore fluorescence decreases as a linear function of light emitted when isolated photophores are stimulated to luminesce with epinephrine, norepinephrine or KCN. Thus it appears that photophore fluorescence is a useful indicator of the presence of luciferin and of ability to luminesce.

In this paper we correlate the distribution of luminescent and non-luminescent forms of the midshipman with the distribution of *Vargula tsujii,* show that feeding with *Vargula* induces luminescence capability in non-luminescent *Porichthys,* and demonstrate that fish thus treated have behavioral control of their newly acquired luminescence.

MATERIALS AND METHODS

Field studies

Specimens of *P. notatus* were taken from nine sites along the Pacific coasts of the United States and Canada (Table I, Fig. 1). Bottom trawls were made at 1–3 knots at depths from 5–500 m. Experimental fish were maintained in separate 40 l aquaria in the laboratory and supplied aerated, sand-filtered sea water at 14°–18°C.

Bioluminescence and fluorescence were measured on fish anesthetized in MS-222 (Sigma), 0.1 g/l sea water, applied until cessation of swimming (2–15 min). Photophore fluorescence was estimated using a 365-nm-emitting u.v. lamp (UVSL 25, Ultra Violet Products. Inc., San Gabriel, Ca.) Field recordings of luminescence were made from anesthetized fish after I.P. injections of 0.1 ml of 0.001 mM DL-arterenol HCl (Sigma) in fish saline (Young, 1933). During the 15 min after injection luminescence was measured continuously with a photomultiplier (EMI 9781B) positioned over the ventral thoracic photophores.

Laboratory studies

Luminescence was recorded in the laboratory by injecting fish as above or subcutaneously, and viewing either all ventral photophores or those at the subcutaneous injection site with a photomultiplier (EMI 9781B). Photomultiplier output was led to a Keithley 427 current amplifier and displayed on a polygraph (Grass, model 79D9).

Luminescence intensity was determined relative to an arbitrary standard with this apparatus, as shown in Figure 2. Bioluminescence was recorded for several

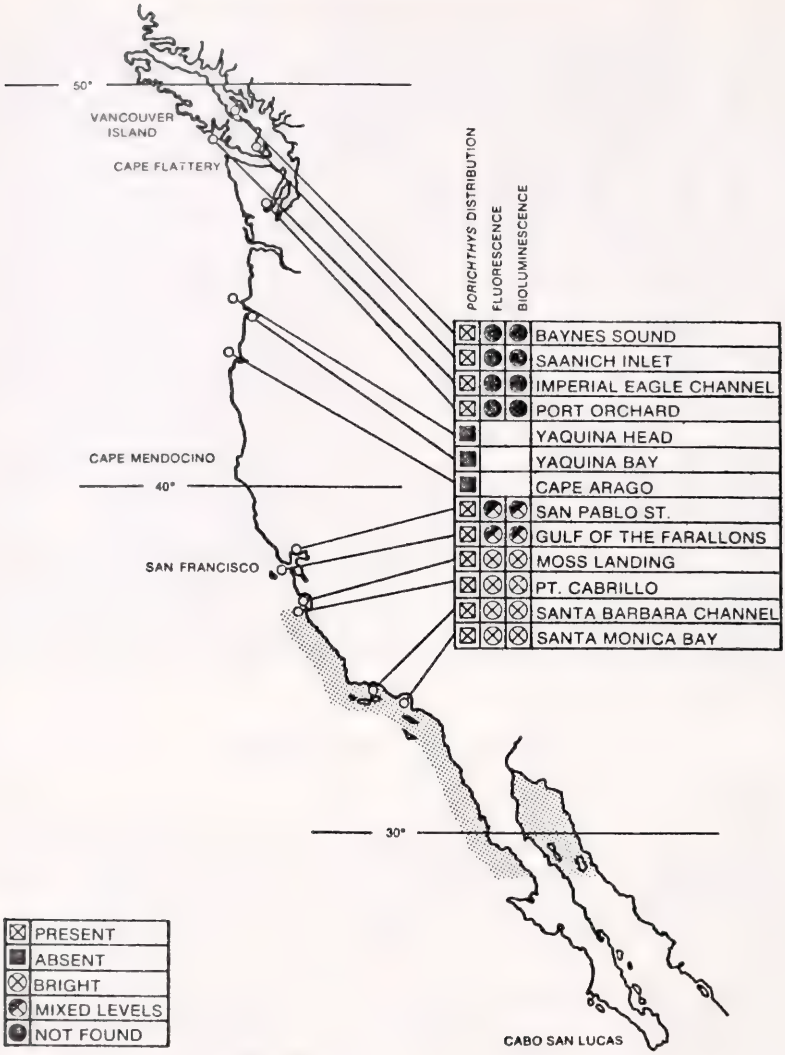

50°
VANCOUVER ISLAND
CAPE FLATTERY
PORICHTHYS DISTRIBUTION
FLUORESCENCE
BIOLUMINESCENCE
BAYNES SOUND
SAANICH INLET
IMPERIAL EAGLE CHANNEL
PORT ORCHARD
YAQUINA HEAD
YAQUINA BAY
CAPE ARAGO
SAN PABLO ST.
GULF OF THE FARALLONS
MOSS LANDING
PT. CABRILLO
SANTA BARBARA CHANNEL
SANTA MONICA BAY
CAPE MENDOCINO
40°
SAN FRANCISCO
30°
CABO SAN LUCAS
PRESENT
ABSENT
BRIGHT
MIXED LEVELS
NOT FOUND

FIGURE 1. Collecting localities showing presence or absence of *Porichthys notatus* and the extent to which they exhibit luminescence and ultraviolet excited fluorescence. Shaded zone indicates distribution of *Vargula tsujii*.

minutes beginning immediately after a local subcutaneous injection of 0.4 ml of 0.001 nM DL-arterenol. A conical light guide, masked so as to limit recording to a single photophore of average size, served to control for the large variation in size of fish and photophores.

Feeding experiments

In attempts to induce luminescence by manipulating the diet, 15 (12–21 cm) non-luminescent fish from Saanich Inlet and Imperial Eagle Channel, Vancouver Island, Canada, were fed parts or whole individuals of several luminescent organisms sympatric with the southern, luminescent *P. notatus*. Organisms, details of preparation and effects on luminescence are shown in Tables II and III.

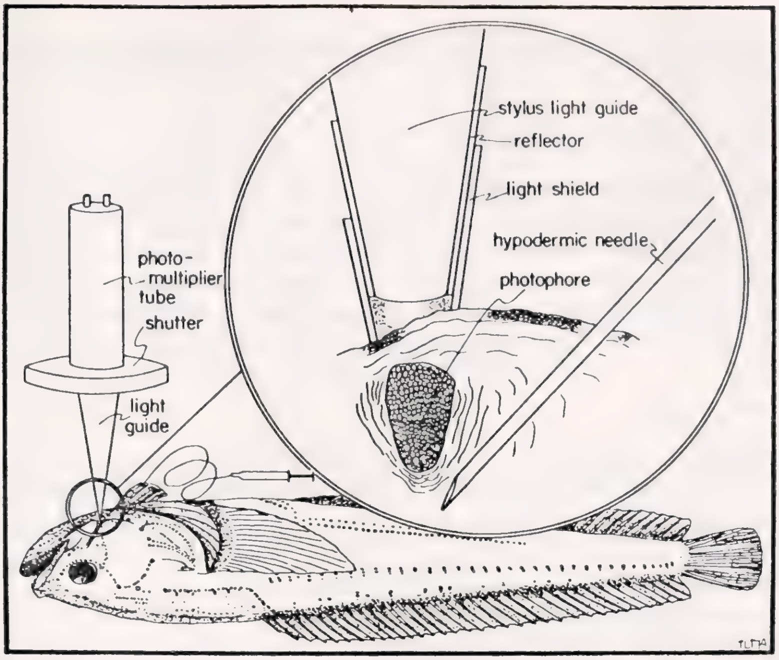


FIGURE 2. Arrangement used in recording luminescence from a single photophore in an intact *Porichthys.*

Anesthetized fish were fed by inserting an appropriate size gelatine capsule of food deep within the esophagus. Close observation during recovery from anesthesia guarded against regurgitation, a rare occurrence. Each feeding greatly exceeded

TABLE II

Materials used in luminescence induction experiments

Organism	Source	Method of preparation
Gonyaulax polyhedra	Cultures supplied by Dr. B. M. Sweeney	Concentrated by low speed centrifugation during night phase
Renilla kollikeri	Santa Barbara Channel	5 mm wide strip from edge of rachis
Vargula hilgendorfii	Vicinity of Chiba, Japan, *via* Dr. F. H. Johnson	Probably air dried. Samples used were brilliantly luminescent when wetted
Vargula tsujii	Collected at underwater light off dock at Catalina Marine Laboratory	Lyophilized after freezing alive and stored over dessicant
Gaussia princeps	Midwater trawls in San Clemente Basin	Fed alive to test fish immediately upon capture
Euphausia pacifica	Same	Same
Gennadus sp.	Same	Same
Ophiopsila californica	Naples Reef, Santa Barbara, California	Arm sections fed fresh to test fish
Stenobrachius californica	Midwater trawls in San Clemente Basin	Frozen alive. Ventral, photophore-containing parts fed after brief thawing

the weight of *V. hilgendorfii* required to induce luminescence capability in non-luminescent fish.

Studies on fluorescence induction

Rate of induction of fluorescence in non-luminescent fish was estimated on one non-luminescent fish (19.5 cm total length) from Bamfield, B. C., fed 361 mg of dried *V. hilgendorfii.* Subsequently three photophores were removed each day and examined with a fluorescence microspectrophotometer (NanoSpec/10S, Nanometrics Inc., Sunnyvale, Cal.) Excitation illumination was from a mercury lamp filtered with UG 1 and BG 12 filters. Fluorescence was recorded at 524 nm.

Behavioral studies

Luminescent responses to mechanical, visual, and electrical stimuli were studied both in luminescent fish from Southern California and in one luminescence-induced fish from north of Cape Flattery, Washington. Single fish were placed in a small

TABLE III

Results of dietary luminescence experiments.

Fish number	Day	Food	Test for luminescence
1	0	*Gonyaulax polyedra*	(−)
	2	*G. polyedra*	
	4	*G. polyedra*	
	9	*G. polyedra*	
	17		(−)
	32	Beef Liver	(−)
	47		(−)
	53	*Vargula higendorfii* (312 mg*)	(−)
	71		(+)
2	0	*Renilla kollikeri*	(−)
	4	*R. kolliekeri*	(−)
	15	*R. kollikeri*	(−)
3	0	*Vargula hilgendorfii* (291 mg*)	(−)
	8		(+)
	12	Beef Liver	(+)
	36		(+)
	142		(+)
	0	*Vargula tsujii* (3**)	(−)
	7		(−)
	39	*V. tsujii* (110 mg*)	
	44		(+)
	75		(+)
	107		(+)
	140		(+)
5	0	*Vargula tsujii* (10 mg*)	(−)
	21	*V. tsujii* (63**)	(+)
	70		(+)
6	0	*Gaussia princeps* (317 mg*)	(−)
	25		(−)
7	0	*Gaussia princeps* (28**)	(−)
	1	*G. princeps* (24**)	
	9		(−)
	23		(−)
8	0	*Gaussia princeps* (12**)	(−)
	1	*G. princeps* (30)	
	9		(−)
	23		(−)
	72	*Euphausia pacifica* (28**)	(−)
	73	*E. pacifica* (14**)	
	74	*E. pacifica* (16**)	
	81		(−)
	95		(−)
	103	*Vargula hilgendorfii* (273 mg*)	(−)
	121		(+)
9	0	*Gaussia princeps* (15**)	(−)
	9		(−)
	23		(−)
	72	*Euphausia pacifica* (35**)	
	73	*E. pacifica* (15**)	
	74	*E. pacifica* (16**)	
	81		(−)
	95		(−)
10	0	*Euphausia pacifica*	(−)
	4		(−)
	19	*E. pacifica*	(−)
	38		(−)
	53		(−)
11	0	*Euphausia pacifica*	(−)
	7	*E. pacifica*	(−)
	12	*E. pacifica* + Beef Liver	
	30		(−)
	41	*Vargula hilgendorfii* (343 mg*)	(−)
	51		(+)
12	0	Beef Liver	(−)
	23		(−)
	38	*Stenobrachius californica*	
	50	*Euphausia pacifica*	(−)
	67	*E. pacifica*	(−)
	75	*S. californica*	(−)
	82	*E. pacifica*	(−)
	119		(−)
13	0	*Gennadas sp* (4***, 3 cm animals)	(−)
	9		(−)
14	0	*Gennadas sp* (4, 3 cm animals)	(−)
	9		(−)
	23		(−)
	72	*Euphausia pacifica* (25**)	
	73	*E. pacifica* (13**)	
	74	*E. pacifica* (16**)	
	81		(−)
	95		(−)
15	0	*Ophiopsila californica*	(−)
	20		(−)

* Weight of lyophilized animals. *V. hilgendorfii* was probably sun dried in Japan and not lyophilized.

** Numbers of living animals, other than *V. tsujii*, which had been lyophilized.

*** Number of 3 cm animals.

aquarium entirely within the view of an upward-facing photomultiplier tube (EMI 9781B). Mechanical stimulation was accomplished by a solenoid-driven rod tapping against the aquarium. Photic stimuli were of two sorts. One was a collimated beam from a 1.5 V incandescent lamp directed across the aquarium at right angles to the photomultiplier field of view. The second was one of two models of the photophore pattern of a 14-cm midshipman. One model presented the lateral aspect and the other a ventral view of the fish. These luminous patterns were produced by templates placed over two green-emitting Sylvania "Panelescent Nightlights."

Low voltage A.C. delivered between silver wire grids at each end of the aquarium invariably induced luminescence in luminescence-competent fish and was used as a control test when other methods of stimulation failed.

RESULTS

Evidence for a discontinuous distribution of P. notatus

P. notatus has been described as ranging from Sitka, Alaska, to Baja California (Hart, 1973). The twelve sites listed in Figure 1 and Table I were sampled to establish regions within this species distribution where luminescence occurs. Figure 1 and Table I show presence or absence of fish and the presence or absence of bioluminescence and fluorescence in collected fish. We were unable to find *P. notatus* between Cape Flattery and Cape Medicino. The existence of this hiatus within the distribution of *P. notatus* was confirmed from two other sources. The first, a survey of museum collections, revealed hundreds of specimens of the midshipman from Puget Sound and Southern California waters but only four specimens collected between Cape Mendicino and Cape Flattery. These were two specimens from Winchester Bay, Oregon (Los Angeles County Museum of Natural History and Oregon State University Ichthyological Museum), one from off the mouth of the Columbia River, and one from Coos Bay, Oregon (O.S.U. Ichthyological Museum). The second confirmation came from a series of 660 trawls along the 100-fathom line between Oxnard, California, and Cape Flattery, Washington, whose results were made available by the NOAA Northwest and Alaska Fisheries Center (Rock Fish Study, 1977, unpublished). *P. notatus* was common from Oxnard to approximately Cape Mendicino. No specimens were recorded between Cape Mendicino and Cape Flattery. Thus the prevalence of *P. notatus* in Puget Sound and in Southern California waters is in striking contrast to its paucity along the Oregon coast.

Correlation of fluorescence with bioluminescence in natural populations

As indicated in Table I and Figure 1, fish from the areas examined in this study were tested for the correlation of fluorescence and bioluminescence. All fish able to luminesce were fluorescent. Monterey coastal waters are evidently the northernmost limit of the entirely luminescent population, since 53 fish from the San Francisco region included non-luminescent (8%) and weakly luminescent (38%) as well as normally luminescent fish (54%). All non-luminescent fish from the San Francisco region were non-fluorescent while those with subnormal luminescence had less than maximal fluorescent capacity. North of the Oregon hiatus neither fluorescence nor bioluminescence was seen. South of Monterey, all fish examined were strongly fluorescent and bioluminescent.

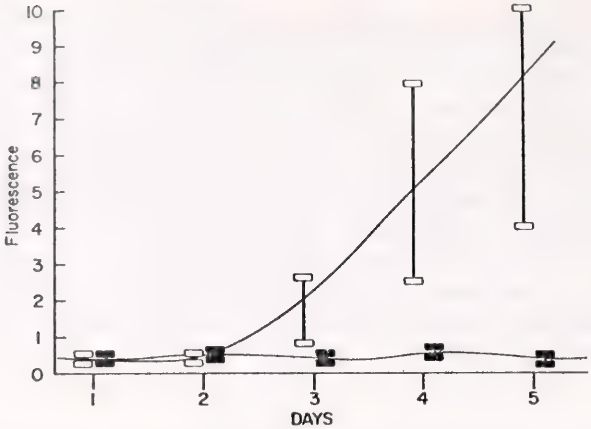


FIGURE 3. The development of fluorescence in a non-luminescent *Porichthys* after feeding with *Vargula.* Each point represents the average of three isolated photophores measured three times. Vertical bars = standard deviation. Upper curve, *Vargula* fed; lower curve, unfed non-luminous fish. Fluorescence scale arbitrary with 10 approximately equal to the typical fluorescence of a southern *Porichthys.*

Fluorescence induction

Onset of photocyte fluorescence was followed in a non-luminescent 19.5-cm fish after feeding with 361 mg of *V. hilgendorfii.* Little or no fluorescence was recordable spectrofluorometrically from isolated photophores before the third day post-feeding. Fluorescence increased markedly from the third to fifth days, when it exceeded the calibrated range of the instrument. During this period the control fish showed no significant amount of photophore fluorescence (Fig. 3).

Dietary induction of luminescence

None of 13 non-luminescent midshipman fish became luminescent when fed upon specimens of eight luminescent organisms sympatric with the Southern California midshipman, as noted in Tables II and III. The four survivors at the end of this experiment were fed approximately 300 mg each of *Vargula hilgendorfii* and all became capable of luminescence upon injection of noradrenaline. In a subsequent experiment, one 19-cm nonluminescent midshipman was fed 110 mg (dry weight; about 700 specimens) of *Vargula tsujii* and developed bright fluorescence and bioluminescence, first noted 4 days after feeding. The ability to luminesce persisted in this fish until its death 120 days later. In this experiment a second nonluminescent, 17.5-cm fish shared the same seawater as the induced fish but was fed beef liver. It developed neither fluorescence nor capacity to luminesce during the induction period for the specimen fed *V. tsujii.* At the conclusion of the experiment this control fish was fed 10 mgs (dry weight; 63 specimens) of *V. tsujii.* Between 15 and 21 days later fluorescence was apparent and the fish luminesced in response to noradrenaline injection. Luminescence capacity persisted until death of the fish 70 days later.

Luminescent capability and behavior

Although Barnes *et al.* (1973) had shown that northern midshipman fish after dietary luminescence induction could be induced to luminesce by electrical stimula-

TABLE IV

Luminescent responses to stimuli

Stimulus type	Number of tests	Percent response	Response characteristics		
			Latency of response (ms)		Duration (seconds)
			Average	Minimum	
A. Southern *Porichthys* (12 fish)					
Light beam	111	26	1300 ± 290*	1,000	5.58 ± 3.50
Model fish	113	30	1030 ± 180	500	4.43 ± 2.36
Mechanical	102	62	660 ± 180	200	7.46 ± 5.40
Electrical	13	84	—	—	11 64 ± 8.41
B. Northern *Porichthys* (1 fish) (First series 82 days after luminescence induction by feeding *Vargula tsujii*)					
Light beam	5	33	1110 ± 160	900	3.41 ± 1.30
Mechanical	12	74	767 ± 98	500	12.00 ± 7.21
(Second series on same fish, 118 days after induction)					
Model fish	19	0	Fish appeared to be blinded, with cornea opaque, perhaps due to fungal attack		
Mechanical	14	71	760 ± 210	300	5.15 ± 2.07

* ± = Standard deviation.

tion, indicating the persistence at least of normal peripheral neural pathways, it is of the greatest interest to determine if the induced northern fish has behavioral control over its artificially endowed capacity to luminesce. To partially answer this question the responses of normally luminescent fish and induced fish to four stimulus types were compared. In order of increasing effectiveness in evoking bioluminescence these were: 1) a midshipman photophore model, 2) a light beam passed through the aquarium, 3) tapping the aquarium side and 4) an A.C. current passed through the aquarium. Table IV sets forth the results of these experiments.

After preliminary tests showed one induced fish to respond to mechanical stimulation, a second fish was carefully examined. Tested with light beam and mechanical stimuli, its responses appeared to fall within the range of normally bioluminescent fish with respect to response latency and rise time (Table IV, Fig. 4). First tested 82 days after an inductive feeding, this fish was left undisturbed and without feeding for an additional 36 days, when its bioluminescent responses to light and mechanical stimuli were found to be still intact. Immediately afterwards it expired following a forced feeding.

DISCUSSION

Discovery that the luminescent ostracod *Vargula tsujii* induces luminescence when fed to nonluminescent *Porichthys* further supports the possibility that a dietary deficiency causes the non-luminescent state of northern *Porichthys*. This

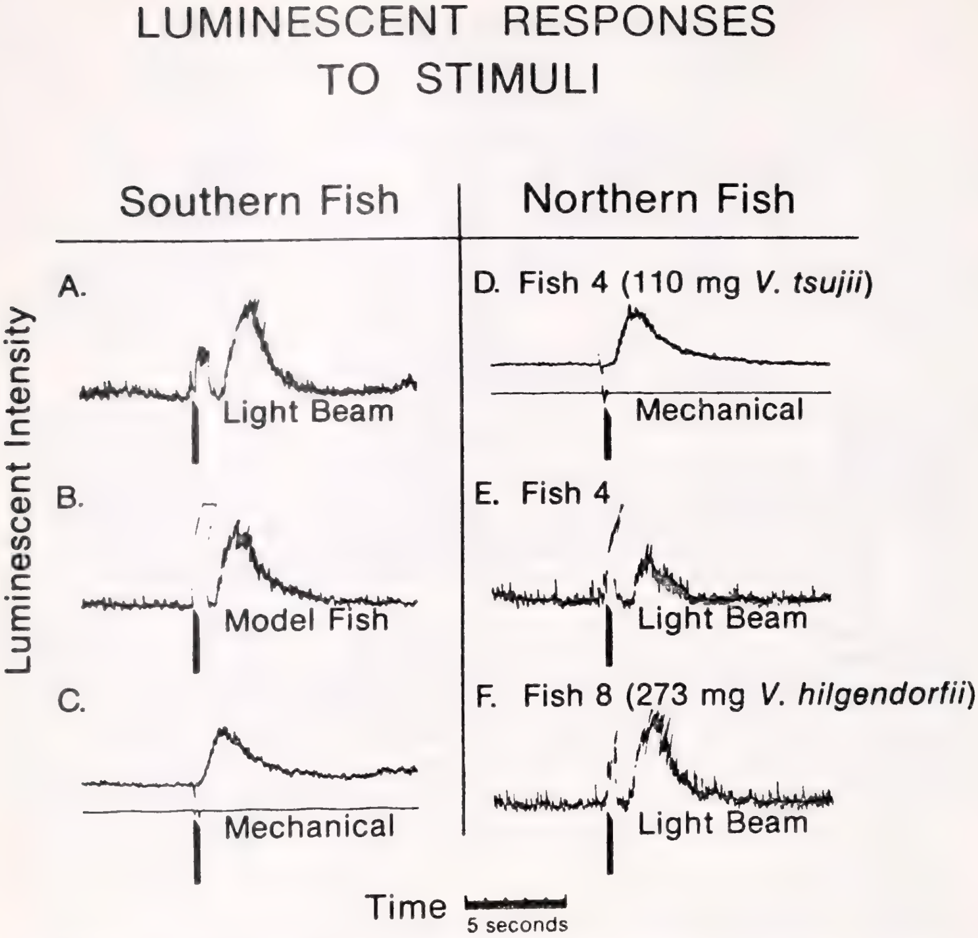


FIGURE 4. Photomultiplier records of behavioral luminescent responses of normally luminescent southern *Porichthys* and *Vargula*-induced northern *Porichthys*.

possibilty is strengthened by the congruence in distributions of the luminescent *Porichthys* population and that of *V. tsujii,* whose northern limit is Monterey Bay (Kornicker and Baker, 1977). This is just south of the zone of incompletely luminescent fish. They also report *V. tsujii* occurring as far south as Bahia de Los Angeles, Baja California, which roughly corresponds to the southern limit of *P. notatus,* according to Miller and Lea (1972). However, no *Porichthys* from waters south of Santa Monica Bay were tested for luminescence in this study.

It is interesting to note that Greene (1899) reported two non-luminous *Porichthys* taken by hook from deep water off Monterey Bay.

Such observations on the overlap in distribution of *V. tsujii* and luminescent populations of *Porichthys,* as well as the inductive capacity of *V. tsujii* when administered to nonluminescent midshipman fish, suggest that all *Porichthys notatus* owe their luminescence to a dietary source of luciferin. However, proof requires evidence of *V. tsujii* or a similarily inductive food source in the *Porichthys* diet and demonstration, by depletion experiments or other means, that this food source is essential to luminescence in fish of the southern population. With respect to the first point, *V. tsujii* has been taken from the gut of the pomocentrid, *Chromis atrilobata* (Kornicker and Baker, 1977), a fish with feeding habits similar to *P. notatus* (personal communication, A. W. Ebeling, University of California, Santa Barbara) and other ostracods have been found in gut contents of *P. notatus* (Ibara, 1967). It therefore seems possible that *V. tsujii* is part of the diet of the midshipman fish, especially during the juvenile phase when both occupy inshore sandy bottoms.

The fact that the peritoneum was darkly pigmented in all fish examined, including post-larvae as small as 30 mm total length, is also suggestive that the midshipman includes luminescent organisms in its diet. McCallister (1967) proposed that such pigmentation protects against being rendered conspicuous by luminescing food in the digestive tract.

Attempts to deplete luminescence capacity in normally luminescent adult fish have not been successful, even though they have been maintained on beef liver or even starved in laboratory aquaria for more than 5 months (Barnes *et al.,* 1973). In view of the large luciferin stores in body tissues it may be that the southern fish have accumulated more than adequate reserves for life upon reaching maturity. Also they may be able to resynthesize or totally synthesize luciferin. An indication that some dietary luciferin is necessary comes from the fact that it is possible to deplete luciferin reserves sufficiently in southern midshipman larvae evidently to permanently abolish luminescence in specimens kept in a sand and activated-charcoal-filtered seawater supply (Case and Warner, unpublished observations).

Haneda *et al.* (1969) proposed that *V. hilgendorfii,* an ostracod of Japanese waters, is a source of luciferin for *Apogon ellioti* and *Parapriacanthus ransonneti.* They cite the following evidence: 1) all three species have similar luciferins and their luminescent systems cross-react; 2) all three species are sympatric; 3) about 12 *V. hilgendorfii* were found in gut contents of a total of more than 1000 *A. ellioti;* and 4) between gut and light organs in both species of fish there is an opening that is the proposed route of movement of dietary luciferin from gut to luminescent tissues. Luciferin is evidently the only component of the luminescent system that might be required from the diet since the luciferase of *V. hilgendorfii* differs from that of *A. ellioti* (Tsuji and Haneda, 1966).

The arguments in favor of dietary dependence of the midshipman luminescent system are similar to those just listed. Failure to identify *V. tsujii* in the midshipman diet may be only a matter of the small number of specimens examined (recall that only 12 *V. hilgendorfii* were found in 1000 *Apogon*) and the fact that *V. tsujii* was not known at the time of Ibara's (1967) study. Lack of a direct connection between gut and luminescent tissues in the midshipman is not an insurmountable impediment to the hypothesis since the wide distribution of photophores in the midshipman makes such a transport mechanism anatomically improbable. Moreover, there seems to exist a concentrating mechanism appropriate to the midshipman's photophore pattern, namely the microvillous borders of its photocytes. These may be specializations for the uptake of luciferin from the demonstrated large body stores (Anctil, 1979; Case and Strause, 1979; Tsuji *et al.,* 1971).

Our work shows that small amounts of *V. tsujii* induce luminescence capacity which persists for long periods, suggesting that relatively infrequent feeding could maintain sufficient luciferin levels in nature. This finding confirms observations by Barnes *et al.* (1973) on the feeding of *Vargula hilgendorfii* to northern midshipman fish.

The amount of luciferin required to induce luminescence capability in the northern midshipman is vanishingly small. Only 9 μg of purified *Vargula hilgendorfii* luciferin induced prolonged luminescence capability in an adult fish weighing approximately 59 gm (Barnes *et al.,* 1973). In the present study 10 mg of dried *Vargula hilgendorfii* fed to a fish of 74 gm (final frozen weight) was effective. Although the ineffectiveness of oxidized luciferin and several luciferin derivatives in induction of luminescence capacity (Barnes *et al.,* 1973; Tsuji *et al.,* 1975) suggests that the luciferin molecule is not recycled, in the absence of more direct evidence this possibility cannot be excluded since there are other plausible explanations of the present results. For example, enzyme induction might occur, triggered by luciferin. Or the luciferin accumulating mechanism that possibly is associated with the photocyte microvillous cell membranes (Case and Strause, 1979; Anctil, 1979) might be uniquely activated by luciferin and not by the derivatives tested. If the latter is true, a recycling mechanism confined to the photocytes could not be revealed by systemic administration of metabolic intermediates.

It is difficult to explain the origin of this possible dietary dependence of *Porichthys* on *Vargula* for luciferin. Despite the reported dissimilarity of fish and ostracod luciferases, it is worth considering that the fish luminescent system was initially obtained in its entirety from the ostracod. Subsequent, loss of ability to synthesize luciferin would not be as serious a matter as loss of ability to synthesize the enzyme, owing to the undoubtedly greater difficulty of assimilating an intact protein from the digestive tract. Thus the present state with dependence only on exogenous luciferin might arise.

A major argument against this total-initial-assimilation hypothesis is that the fish would have obtained the biosynthetic elements of the luminescent system before developing photophore and control mechanisms. While it might be possible to conjecture some adaptive value for this interim state, it would seem to be the simpler hypothesis to propose that both the ancestral midshipman and *Vargula* independently evolved luminescent systems which underwent sufficient biochemical convergence to allow the *Vargula* luciferin to function in the midshipman system.

Resolution of the origins of this possible dietary dependence, as well as those suspected in other luminescent systems, would be materially advanced by amino-acid sequencing of the relevant luciferases. While certain of these are distinguishable (Tsuji and Haneda, 1966), the techniques used—chromatography, enzyme kinetics, and immunological studies—do not eliminate the possibility that homologous amino-acid sequences exist among these enzymes.

The 1110-km apparent hiatus along the northwest U. S. coast may stem from the low summer temperature of inshore waters in this region, where there is a summer upwelling of cold water (C. Bond, Oregon State University, personal communication). Average summer temperatues at 10 m along the Oregon coast are about 9°C, which is to be compared with 15°C at Vancouver Island and 15°–18°C for outer San Francisco Bay and the more southern waters covered in this study (Barkley, 1968). Migration of *Porichthys* from deeper water to inshore nesting sites (Arora, 1948) coincides with the onset of upwelling so this cold water might interfere with nesting, courtship, or embryonic development. This does not, however, eliminate the possibility of genetic exchange between northern and southern populations; for unless fish return to the coastal zone of origin to breed, they might intermix during their sojourn in deep water. We do not have enough data to decide whether the midshipman occurs at depths greater than 200 m off the hiatus zone. In other localities they are reported down to 600 m, and a fisherman at Eureka, Calif., told us of trawling midshipman fish near there at 1000 m.

There is no obvious causal relationship between the distributional hiatus and the wholly non-luminescent population to its north because the non-luminescent state clearly is found in the Monterey to San Francisco Bay region. Considering the geological history of the northwestern continental margins, it would seem plausible to suppose that *Porichthys,* a member of a predominantly tropical family, colonized northern waters during the present warm period or during the last interglacial, about 120,000 years ago. During this period $^{18}O/^{16}O$ ratios in deep sea fossils indicate that water temperatures were several degrees higher than now (Shackleton and Opdyke, 1976). Perhaps breeding populations of the midshipman were continuous along the west coast during this or an earlier warm period. Similarly, *Vargula* may have had a distribution matching that of the midshipman. After establishment of the cooling trend in the northeast Pacific, *Vargula* may have retreated to its present distribution, perhaps contemporaneously with establishment of the *Porichthys* coastal hiatus.

Interesting questions are raised by persistence in the northern population of a luminescence system rendered nonfunctional for lack of luciferin but otherwise morphologically, biochemically, physiologically, and behaviorally intact. Assuming that there is no interchange of individuals between northern and southern populations, there should be little advantage in retaining the non-functional luminescence system by the northern fish. Yet, as we find, even appropriate behavioral control persists. Our observations cannot be interpreted as persistence of anything other than a specific bioluminescence behavioral control system. For example, a nonspecific, alarm-type adrenergic arousal is unlikely since bioluminescence is difficult to evoke in the midshipman by violent nonspecific stimulation, as first noted by Greene (1899). Since it is doubtful that the resource investment required for development and maintenance of a nonluminescent system would continue for long without some value to the organism, we suggest that this state is either a recent evolutionary development or that the luminescent system has importance beyond the generation of light. For example, the visible photophore pattern may be a necessary intraspecific recognition sign.

Bioluminescence has been nominated to serve at least 20 biological functions (Buck, 1978). *Porichthys* is implicated in at least three of these: mimicry, warning, and courtship. McCallister (1967) notes references to the possibility that the photophore pattern of the midshipman mimics the luminescence of a ctenophore. Tsuji *et al.,* (1971) suggest that the *Porichthys* pattern of luminescence resembles the light of a small swarm of euphausids. This might allow undetected approach to such a swarm, attract the swarm to the fish, or provide protective mimicry against its own predators. Lane (1967) describes the use of luminescence in aggressive behavior of *Porichthys porosissimus.* Crane (1965) observed a courtship display in an aquarium in which grunting and flashing by a male followed its exposure to a female artificially induced to bioluminesce. The similarity of the bioluminescence emission peak and the wavelenth of maximal visual sensitivity in the midshipman further supports the possibility that bioluminescence is of intraspecific significance (Fernandez and Tsuji, 1976). In addition to these possible behavioral uses it must be obvious that the characteristic, predominantly ventral distribution of photophores in the midshipman evokes the possibility of counterillumination (Clarke, 1963).

We are in no position to confirm any of these proposed functions and, further, we note the curious fact that these fish live and reproduce successfully in Puget Sound and even are found in the open waters off outer Vancouver Island with no luminescence capability. This alone places in question any necessary role in courtship. The other proposed roles might still be reasonable since the Puget Sound environment may differ sufficiently from the open marine environment of luminescent-competent midshipman fish to allow speculation that defensive or counterillumination uses of bioluminescence would be unnecessary there and yet vital in the open sea. The outer Vancouver Island fish might seem to defeat even these arguments for a role for bioluminescence. However, it is possible that this population is not truly established there but is maintained by recruitment from inner Puget Sound. Finally, it must be noted that some behavioral modification in the Puget Sound fish, such as a change from nocturnal to diurnal activity, might have diminished the adaptive value of luminescence. Under such conditions the photophore pattern might still be of value, as suggested above. Nevertheless, north of Cape Flattery, *Porichthys* flourishes while lacking the luminescent capacity which presumably adds to the fitness of its southern counterpart.

We are indebted to the following for much valuable advice and assistance: Peter Anderson, Shane Anderson, B. M. Sweeney, R. Satterlie, B. Sumida, and R. Winquist, all of University of California, Santa Barbara; Milton Boyd, Humboldt State University; T. Black, University of British Columbia; G. Calliet, Moss Landing Marine Laboratory; B. B. Collette, National Marine Fisheries Service; Robert Givens, University of Southern California; Donald Gunderson, University of Washington; the late Carl Hubbs, Kenneth H. Nealson, and Frederic I. Tsuji, Scripps Institution of Oceanography; Frank H. Johnson, Princeton University; E. D. Lane, Environment Canada; Robert Lavenberg and C. Swift, Los Angeles County Museum of Natural History; Carl Liem, Museum of Comparative Zoology, Harvard University; John E. McInerney, Bamfield Marine Laboratory; Brad Meyers, Los Angeles; Robert Morris, University of Oregon; A. Paden, British Columbia Provincial Museum; C. Levitt Smith, American Museum of Natural History; Robert Tasto and Paul Riley, California Fish and Game; and Arthur D. Welander, University of Washington.

Our research was supported by Office of Naval Research Contract NOOO14-75-C-024 and University of California Faculty Research Funds.

SUMMARY

Non-luminous northern midshipman fish, members of the species *Porichthys notatus,* are shown to exist as two disjunct populations, one previously known from the Puget Sound area, and the other in waters near San Francisco, where nearly half of the fish collected are non-luminescent or exhibit diminished luminescent capacity. The San Francisco population represents the northernmost occurrence of the southern *Porichthys notatus* population, in which non-luminescent fish are rare or absent. Between the San Francisco and Puget Sound populations a zone between Capes Flattery and Mendicino appears to completely lack midshipman fish in near-shore waters. The distribution of the luminescent ostracod, *Vargula tsujii,* corresponds to that of the luminescent southern fish in that its northermost extension is in the Monterey-San Francisco region. Northern, non-luminescent midshipman fish are rendered luminescent by feeding with *Vargula tsujii.* Feeding with seven other luminescent organisms that might contribute to the *Porichthys* diet did not induce luminescence in northern *Porichthys.* The presence of ultraviolet-excited, 524-nm photophore fluorescence was strongly correlated with luminescence capability in both natural populations and experimentally fed fish. Northern midshipman fish in which luminescence capability was induced by feeding *Vargula* were shown to still posses a behavioral luminescence control system indistinguishable from normally luminescent fish. The bearing of these observations on the origin of bioluminescence and its significance in the life of the midshipman fish are discussed.

LITERATURE CITED

ANCTIL, M., 1977. Development of bioluminescence and photophores in the midshipman fish, *Porichthys notatus.* J. Morphol., **151**: 363–395.

ANCTIL, M., 1979a. Ultrastructural correlates of luminescence in *Porichthys* photophores. I. Effects of spinal cord stimulation and exogenous noradrenaline. *Rev. Can. Biol.,* **38**: 67–80.

ANCTIL, M., 1979b. Ultrastructural correlates of luminescence in *Porichthys* photophores. II. Effects of metabolic inhibitors. *Rev. Can. Biol.,* **38**: 81–96.

ANCTIL, M., AND J. F. CASE, 1976. Pharmacological study of denervation induced by 6-hydroxydopamine in *Porichthys* photophores. *Cell Tissue Res.,* **166**: 365–388.

ARORA, H. F., 1948. Observations on the habits and early life history of the batrachoid fish, *Porichthys notatus* Girard. *Copeia,* **1948**: 89–93.

BAGUET, F., 1975. Excitation and control of isolated photophores of luminous fishes. *Prog. Neurobiol. (Oxf.),* **5**: 97–123.

BAGUET, F., AND J. F. CASE, 1971. Luminescence control in *Porichthys* (Teleostei): Excitation of isolated photophores. *Biol. Bull.,* **140**: 15–27.

BAGUET, F., AND A. M. ZIETS-NICOLAS, 1979. Fluorescence and luminescence of isolated photophores of *Porichthys. J. Exp. Biol.,* **78**: 47–57.

BARKLEY, R. A., 1968. *Oceanographic Atlas of the Pacific Ocean.* University of Hawaii Press, Honolulu. 146 pp.

BARNES, A. T., J. F. CASE, AND F. I. TSUJI, 1973. Induction of bioluminescence in a luciferin deficient form of the marine teleost, *Porichthys,* in response to exogenous luciferin. *Comp. Biochem. Physiol.,* **46**: 709–723.

BUCK, J. B., 1978. Functions and evolution of bioluminescence. Pp. 419–473 in P. J. Herring, Ed., *Bioluminescence in Action.* Academic Press, London.

CASE, J. F., AND L. G. STRAUSE, 1979. Neurally controlled luminescent systems, Pp. 331–365 in P. J. Herring, Ed., *Bioluminescence in Action.* Academic Press, London.

CLARKE, W. D., 1963. Function of bioluminescence in mesopelagic organisms. *Nature,* **198**: 1244-1247.

CORMIER, M. J., J. M. CRANE, JR., AND Y. NAKANO, 1967. Evidence for the identity of the luminescent systems of *Porichthys porosissimus* (fish) and *Cypridina hilgendorfii* (crustacean). *Biochem. Biophys. Res. Commun.,* **29**: 747–752.

CRANE, J. M., 1965. Bioluminescent courtship display in the teleost *Porichthys notatus. Copeia,* **2**: 239–241.

FERNANDEZ, H. R., AND F. I. TSUJI, 1976. Photopigments and spectral sensitivity in the bioluminescent fish, *Porichthys notatus. Marine Biol.,* **34**: 101–107.

GREENE, C. W., 1899. The phosphorescent organs in the toadfish, *Porichthys notatus* Girard. *J. Morphol.,* **15**: 667–696.

GREENE, C. W., AND H. H. GREENE, 1924. Phosphorescence of *Porichthys notatus,* the California singing fish. *Am. J. Physiol.,* **70**: 500–506.

HANEDA, Y., F. H. JOHNSON, AND O. SHIMOMURA, 1966. The origin of luciferin in the luminous ducts of *Parapriacanthus ransonneti, Pempheris klunzingeri,* and *Apogon ellioti.* Pp. 533–546 in F. H. Johnson and Y. Haneda, Eds., *Bioluminescence in Progress. Princeton University Press,* Princeton, New Jersey.

HANEDA, Y., F. I. TSUJI, AND N. SUGIYAMA, 1969. Luminescent systems in apogonid fishes from the Philippines. *Science,* **165**: 188–190.

HART, J. L., 1973. *Pacific Fishes of Canada.* Bull. 180, Fisheries Res. Board of Canada. 740 pp.

IBARA, R. M., 1967. Biology of the midshipman fish, *Porichthys notatus* Girard. M.A. Thesis, University of California, Santa Barbara, California.

KORNICKER, L. S., AND J. H. BAKER, 1977. *Vargula tsujii*, a new species of luminescent ostracoda from Lower and Southern California (Mycopoda: Cypridinae). *Proc. Biol. Soc. Wash.*, **90**: 218–231.

LANE, E. D., 1967. A study of the Atlantic Midshipman, *Porichthys porosissimus*, in the vicinity of Port Aransas, Texas. *Contrib. Mar. Sci.*, **12**: 1–53.

MCCALLISTER, D. E., 1967. The significance of ventral bioluminescence in fish. *J. Fish. Res. Board Canada*, **24**: 537–554.

MILLER, D. J., AND R. N. LEA, 1972. *Guide to the Coastal Marine Fishes of California. Cal. Fisheries Bull., No. 157*, 249 pp.

NICOL, J. A. C., 1957. Observations on photophores and luminescence in the teleost *Porichthys*. *Q. J. Microsc. Sci.*, **98**: 179–188.

SHACKLETON, N. J., AND N. D. OPDYKE, 1976. Oxygen isotope and paleomagnetic stratigraphy of Pacific core V28–239 late Pliocene to latest Pleistocene. *Geol. Soc. Am. Mem.*, **145**: 449–464.

STRUM, J. M., 1968. Fine structure of the dermal luminescent organs, photophores, in the fish, *Porichthys notatus*. PhD. Dissertation, University of Washington, Seattle.

STRUM, J. M., 1969a. Fine structure of the dermal luminescent organs, photophores, in the fish, *Porichthys notatus*. *Anat. Rec.*, **164**: 433–462.

STRUM, J. M., 1969b. Photophores of *Porichthys notatus:* ultrastructure of innervation. *Anat. Rec.*, **164**: 463–478.

TSUJI, F. I., A. T. BARNES, AND J. F. CASE, 1972. Bioluminescence in the marine teleost, *Porichthys notatus*, and its induction in a non-luminous form by *Cyprinda* (ostracod) luciferin. *Nature*, **237**: 515–516.

TSUJI, F. I., AND Y. HANEDA, 1966. Chemistry of the luciferases of *Cypridina hilgendorfii* and *Apogon ellioti*. Pp. 137–149 in F. H. Johnson and Y. Haneda, Eds., *Bioluminescence in Progress*. Princeton University Press, Princeton, New Jersey.

TSUJI, F. I., R. V. LYNCH, III, AND N. SUGIYAMA, 1971. Luminescent cross reactions of *Porichthys luciferin* and theories on the origin of luciferin in some shallow-water fishes. *Comp. Biochem. Physiol.*, **40**: 163–179.

TSUJI, F. I., B. G. NAFPAKTITIS, T. GOTO, M. J. CORMIER, J. S. WAMPLER, AND J. M. ANDERSON, 1975. Spectral characteristics of the bioluminescence induced in the marine fish, *Porichthys notatus*, by *Cypridina* (Ostracod) luciferin. *Mol. Cell. Biochem.* **9**: 3–8.

WILIMOVSKI, N. J., 1954. List of the fishes of Alaska. *Stanford Ichthyol. Bull.*, **4**: 279–294.

YOUNG, J. Z., 1933. The preparation of isotonic solutions for use in experiments with fish. *Pubbl. Stn. Zool. Napoli*, **12**: 425–431.

Reference: *Biol. Bull.*, **159**: 247–257. (August, 1980)

ASPECTS OF THE LIFE HISTORY OF *CARCINONEMERTES ERRANS* (NEMERTEA: CARCINONEMERTIDAE), AN EGG PREDATOR OF THE CRAB *CANCER MAGISTER*

DANIEL E. WICKHAM

University of California, Bodega Marine Laboratory, Bodega Bay, California 94923

The nemertean genus *Carcinonemertes* has been known for over a century as an epibiont of brachyuran crabs. Even though symbiotic life styles are highly unusual for nemerteans, only a limited amount of research has been devoted to the study of *Carcinonemertes*. Worms of this genus infest crabs after a planktonic larval stage. They next remain dormant, encysted on the host's exoskeleton. Worms on female hosts migrate into the host egg clutch when it is oviposited for brooding under the abdomen. There they grow, mature, and lay their own eggs.

While the basic outline of the life history of *Carcinonemertes* has long been known, it was only recently confirmed as a predator of its hosts' eggs (Wickham, 1978). Earlier workers had suspected that *Carcinonemertes* was an egg predator, but never actually observed feeding (Humes, 1942; Kuris, 1971). The worms have been considered generalists in terms of host specificity. But little is known of the extent to which *Carcinonemertes* is capable of affecting host population dynamics.

Carcinonemertes errans Wickham is the first of these worms found to be specific to a single host species: the commercially important Dungeness crab, *Cancer magister* Dana. This worm recently was found to exist at epidemic levels on the Central California Dungeness crab population (Wickham, 1979a). The egg mortality it has caused in this region is high enough (averaging over 50% of the eggs produced annually) to implicate this worm in the collapse of the Central California Dungeness crab fishery. *Carcinonemertes* occurs on a large number of ecologically and economically important brachyuran crabs, and is now known to be capable of having a significant effect on its hosts' reproductive output. The following study on the life history of *C. errans* was initiated for this reason.

MATERIALS AND METHODS

Aspects of the life history of *C. errans* were studied in both the laboratory and the field. Worm abundance in the field was investigated using crabs caught in trawls and traps at Bodega Bay and Eureka, California. These crabs were dissected and all worms observable were counted and their locations on the exoskeleton noted.

Samples also were obtained from the egg clutches of ovigerous female crabs by fishermen in the field. Sampling was done by inserting forceps into the egg clutch approximately at the middle of the exposed portion of the clutch. Care was taken to insert the forceps deeply enough to remove complete egg-bearing setae. The egg samples obtained contained an average of approximately 100 egg-bearing setae which each held about 200 eggs. Dungeness crabs carry up to 2,500,000 eggs in a clutch, so these samples were approximately 1% of the clutch. The egg samples were placed in vials with a 5% formalin sea water solution and examined later with a dissecting microscope.

During examination of the samples all worms were removed and counted. Worm egg strings, which are laid among the crab eggs, were also counted and a portion removed for weighing. The number of crab eggs in each sample was computed by removing a subsample of 10–15 crab-egg setae and counting all live and dead eggs. Percent egg mortality was computed from this and the subsample was dried and weighed to compute the weight per egg. The rest of the sample was then dried and weighed and the weight per egg used to estimate the total number of eggs in the sample. Worm population density was expressed as worms per 1000 crab eggs. Number of worms per host was computed for egg samples obtained from crabs which had been measured for carapace width at the time of sampling. Egg clutch size was estimated for these crabs from the size fecundity curve for the Dungeness crab (Botsford and Wickham, 1978) and worms per 1000 eggs were then used to compute total worms persent in egg clutches.

The actual age of worms present on crabs was unknown, since it was impossible to tell when specific worms infested their hosts. Maturity and essentially all growth of worms living on hosts occurs during the host's egg-brooding period, so the chronology of growth and reproductive processes depends on the number of days worm populations spend in the host's egg clutch. Most worms on hosts migrate to the egg clutch within a day or two after host oviposition, so worm age was expressed as the same age as the crab embryos. Age of crab embryos was estimated by comparing the development of eggs in a sample with descriptions of embryos obtained from crabs sampled throughout their brooding period in the laboratory.

Dry weight of worm populations in egg samples from individual hosts was measured and the average weight per worm computed. Worm fecundity was measured by weighing worm egg strands in the samples. A counted sample of 20,000 worm eggs was dried and weighed to determine the weight per egg and this figure was used to compute the number of eggs per strand. Isolated worms were reared in 100-ml jars with approximately 1000 crab eggs for food until they laid their own eggs, to determine how many egg strands each worm laid in a single host's brooding period. The ratio of worm egg strands to worms was computed from field samples to determine when in the host's brooding period worms produced eggs.

Worm distribution with a host egg clutch was determined by measuring the distance of worms from the tip of the host's egg setae. Distribution of worm egg strings in the host egg clutch also was determined in this fashion.

Results

Early life history

Worm larvae hatch from an egg strand laid among the host eggs. The larva is a simple ciliated ellipse about 100 μm in length, with anterior and posterior apical tufts of what appear to be sensory setae. Upon hatching it immediately swims into the water column and after a few hours begins to alternate crawling on the container bottom with swimming.

Crabs first begin to carry worms after they reach a carapace width of 20 mm (Fig. 1). Worm numbers increase with crab size.

Juvenile worms remained alive for as long as 6 months in finger bowls even though no food was added. The sites of juvenile worm infestation differed between

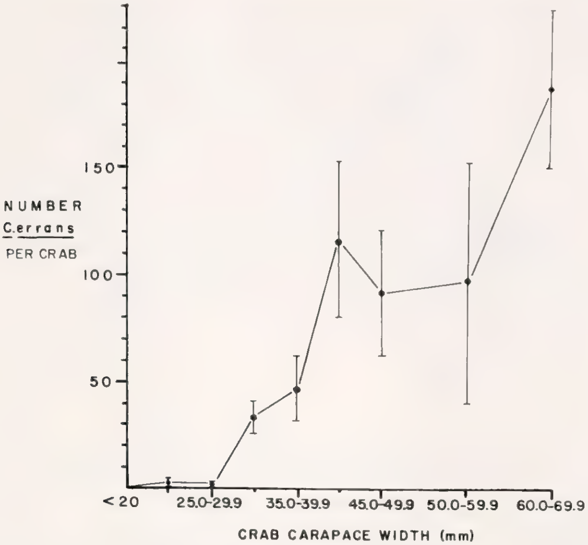


FIGURE 1. Number of *Carcinonemertes errans* per crab (±s.e.) from newly settled juvenile crabs in Bodega Bay, California. N = 59 crabs.

male and female hosts. Dissection of mature, non-ovigerous female crabs revealed worms in virtually every protected crevice or joint on the animal. Areas on the carapace lined with setae harbored large clusters of mucus-covered worms. Groups of worms which, while topologically external, appeared to be internal, could be found in the invaginations of the apodemes. Worms were among the mouth parts, and large populations were found in the eye sockets and on the bases of the eye stalks. The average number on this group of 11 females was 15,011 worms. Some of the sites occupied on these crabs might not be infested at lower densities.

On mature male crabs, the major site of infestation was beneath the abdomen, with concentration often at its base or on the copulatory appendages, although some worms were found elsewhere.

More than 90% of the juvenile worm population on one mature male crab which molted in the laboratory sometime at night had moved off the exuvium onto the new soft exoskeleton by morning.

Host oviposition and C. errans *trophic period*

Juvenile specimens of *C. errans* leave the host's exoskeleton and move into its egg clutch within 1–2 days after oviposition. Worms begin to feed on host eggs within a day of entering the egg clutch.

During the early period of the crab's brooding, worms spend most of their time at the periphery of the egg clutch. Worms are concentrated near the tips of individual egg setae during the first 40 days of host brooding. As they mature they distribute themselves more evenly (Fig. 2).

Feeding *C. errans* move freely about the host egg clutch during the host's entire brooding period. They do not secrete a sheath as do all other described species of *Carcinonemertes*.

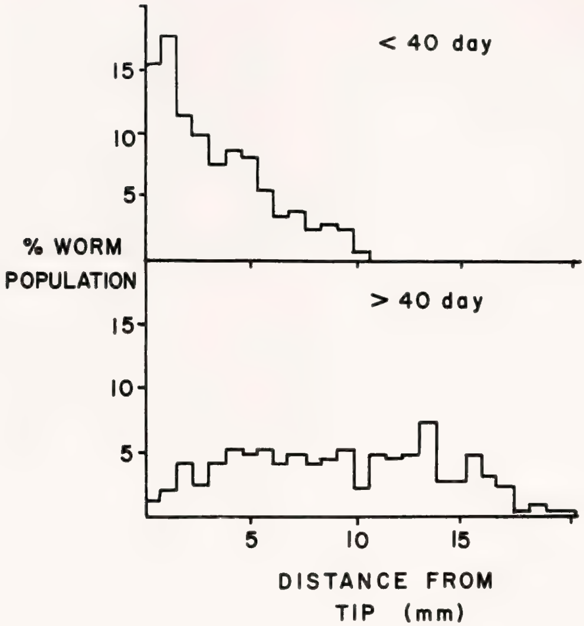


FIGURE 2. Distribution of *Carcinonemertes errans* along crab egg setae for broods less than and greater than 40 days of development. N = 884 worms, < 40 days; 851 worms, > 40 days.

Worm growth

Juvenile worms remain approximately 0.5 mm in length while on the host's exoskeleton. The worms grow only during their feeding period in the host egg clutch. Average worm weight increases slowly through the first 40 days of host brooding but then begins to increase more rapidly during the second half of the ovigerous period (Fig. 3).

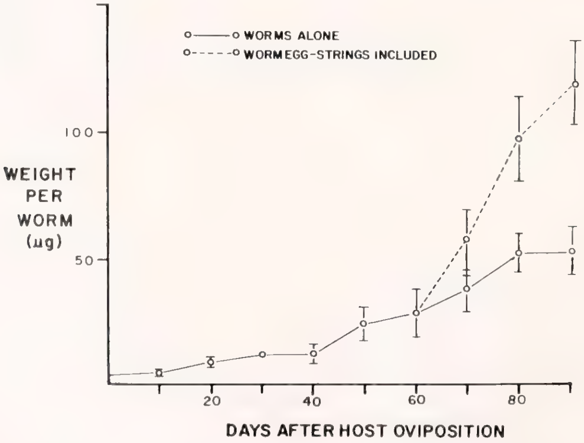


FIGURE 3. Average weight of *Carcinonemertes errans* (±s.e.) as a function of age of their host egg clutch. Dashed line includes the weight of the worm egg strings on a per worm basis. N = 196 crabs.

The worms' growth appears to be sensitive to their densities within individual host egg clutches. Analysis of samples of similiar age for the relationship of average worm weight to density shows that most of the decrease in growth rate occurs as density increases to about 10 worms/1000 crab eggs (Fig. 4).

The appearance of *C. errans* changes as growth and maturity proceed. The sizes of the proboscis chamber and the stylet remain constant (stylet about 50 μm in length) throughout life on the host. As female worms begin to increase in size their epidermises become more transparent as the white spots in the epidermis separate, eventually allowing the gut pouches to become visible along the sides. Female worms take on a segmented appearance when mature, due to the alternation of ovaries and lateral outpockets of the gut. Eggs are fertilized internally and become visible through the epidermis at maturity.

Worm reproduction

Copulation by specimens of *C. errans* was never observed; however, worms are found aggregated in clusters or in pairs from about 20–40 days after host oviposition. Copulation may occur during this period, since several female worms isolated 30 days after host oviposition with approximately 100 crab eggs for food produced fertile eggs.

Worms begin laying eggs after 65–70 days in the host egg clutch. Eggs are laid in a cylindrical gelatinous matrix which is wrapped around the host egg string. Worm egg strands can be found attached all along the host egg setae but are less frequent near the tips at the periphery of the egg clutch (Fig. 5).

Fecundity of *C. errans* is variable and appears to be density dependent. Worms held in the laboratory exhibited high variability in both the number of egg strands produced and the number of eggs per strand (Table I).

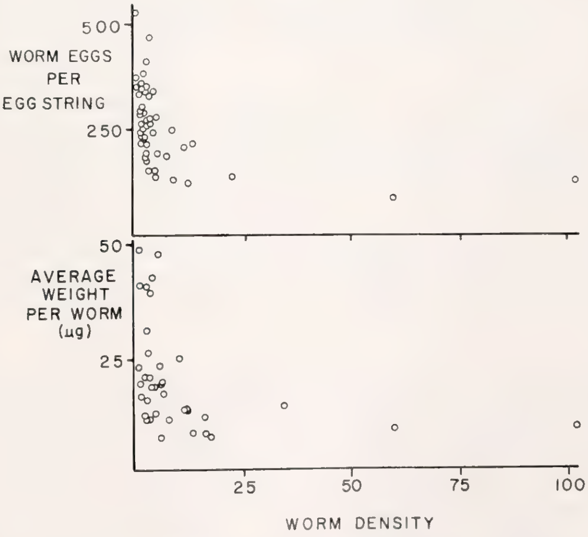


FIGURE 4. Number of worm eggs per egg string and average weight per worm of similarly aged samples as a function of worm density in the egg clutch (worms per 1000 crab eggs). N = 46 samples for eggs per string, N = 37 samples for average weight.

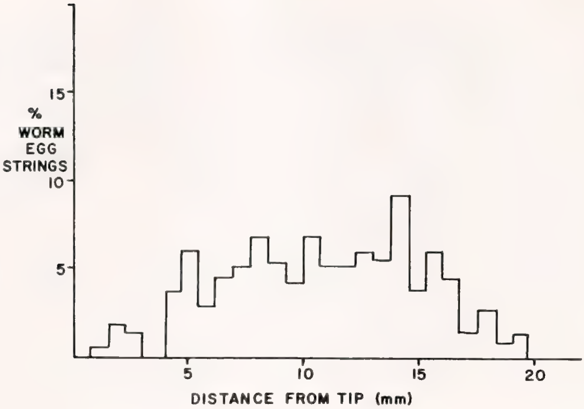


FIGURE 5. Distribution of egg strings of *Carcinonemertes errans* along host egg setae. N = 213 worm egg strings.

Total fecundity per worm declined as the number of worms in 100-ml jars increased, for a limited number of samples (Fig. 6).

In field samples the ratio of worm egg strands to worms ranges up to 7.12. The average was 1.92 egg strands per worm in 73 samples of crab eggs in their last 10 days of development (more than 80 days after host oviposition). The average number of eggs per strand, based on weight measurements of 2500 egg strands, was 265. In these samples the number of eggs per strand declined with density, with most of the decline occurring at the lower densities (Fig. 4).

Infestation rates

Carcinonemertes errans differs from other congeners, being present on over 98% of all potential hosts. All non-egg-bearing crabs above 20 mm carapace width in the Bodega Bay area in Central California had worms. Similarly, only 10 samples from crab egg clutches out of more than 500 collected over a 5-year period contained no worms.

Worm density (worms per 1000 crab eggs) in samples varied from 0 to 101.8 in one sample collected outside San Francisco Bay. Average worms per 1000 crab eggs for seasonal crab-egg collections from Pacific coast localities varied from a low of 0.66 in Alaska in 1979 to 25.41 just outside San Francisco Bay in 1977/78 Table II). Worm density per crab in those collections in which the size of the crabs was measured varied from 1430 in Alaska in 1979, to 20,580 in Washington in 1978/79. Using the average fecundity of the measured crabs it was possible

TABLE I

Number of egg strands produced by worms in the laboratory. The number of egg strands per worm and total fecundity data are based on average values per container. Individual fecundity could only be measured in jars with single worms. N = 29 worms.

	Egg strands per worm	Eggs per egg strand	Fecundity per worm
Average	3.1	446	1339
Range	1–8	79–1409	847–4784

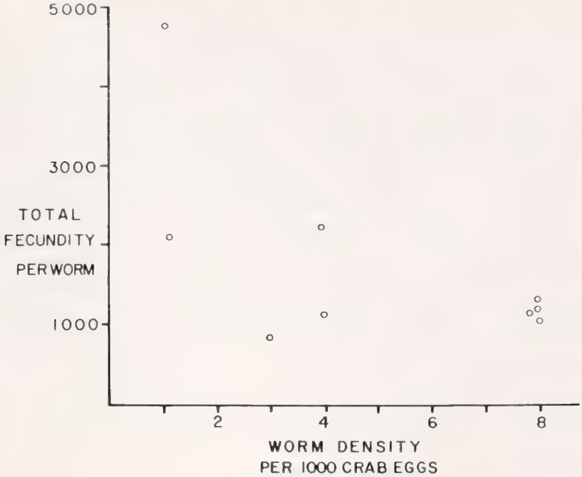


FIGURE 6. Fecundity per worm for *Carcinonemertes errans* held in 100-ml jars at various worm densities. N = 9 samples.

to estimate an average of 43,026 worms per crab outside San Francisco Bay, in 1977/78.

DISCUSSION

The larval life of *Carcinonemertes* is poorly known. Based on the timing between maximum hatching and maximum recruitment to hosts by *C. epialti* on the host *Hemigrapsus oregonensis* by Kuris (1978) and Roe (1979) the larval life

TABLE II

Average worm densities (worms per 1000 crab eggs) for yearly samples along the Pacific coast of North America along with coefficients of variations in density and projected average crab egg mortality from the sampled populations.

Sample collection	Number of samples	Mean worm density	Coefficient of variation	Mean worms per crab (for measured crabs)	Projected % crab egg mortality
Eureka					
1974–75	32	1.78	182.0%		10.6
1975–76	41	4.98	1177.3%		28.5
1977–78	50	4.03	221.1%	6901	30.0
1978–79	35	5.90	425.8%	12,049	43.9
San Francisco					
1974–75	37	14.59	886.6%		63.3
1975–76	86	8.56	1070.9%		50.3
1976–77	44	10.02	823.9%		45.1
1977–78	48	24.96	2905.5%		62.5
1978–79	33	7.76	542.1%		44.6
Washington					
1975–76	16	2.25	338.7%	4647	15.9
1978–79	19	11.14	265.4%	20,580	61.2
Alaska					
1978–79	30	0.66	125.8%	1430	7.6

of this worm species appears to be about 8 months. Juvenile *C. errans* were not found on young-of-the-year Dungeness crabs until August or September, when the newly settled hosts exceeded 20 mm carapace width. Older crabs carried too many worms to accurately count and worms could settle on them at any time during the year. But the worms arriving on young-of-the-year crabs have to be at least 8–9 months old, having hatched the previous Decmber or January at the end of host brooding. *Carcinonemertes* appears to have a relatively long larval period when compared to many other planktonically dispersed marine invertebrates.

Worm numbers increase with crab size during the first few months of the crab's benthic existence. This increase appears to occur throughout the host's life, since adult crabs could accrue in excess of 100,000 worms. Kuris (1978) found a similar increase of *C. epialti* on *H. oregonensis* of increasing size. Since *H. oregonensis* sheds its worms at molting, he proposed that worms settle continuously during the host's intermolt period and larger numbers occurred on larger crabs due to their longer intermolt periods.

Recruitment to hosts by *C. errans* differs from *C. epialti*. *C. errans* juveniles move from the exuvium to the new exoskeleton when the host molts, so hosts can acquire increasing numbers throughout their lives. P. Roe, J. Crowe, L. Crowe, and D. E. Wickham (in preparation) demonstrated that juvenile *C. errans* actively absorbs dissolved primary amines from sea water and that leakage of amines across the uncalcified portion of the host's exoskeleton appears sufficient to meet dormant worms' metabolic needs. It is possible that worms arriving on a female host may remain for several years until the host broods eggs.

The sites of infestation by juvenile *Carcinonemertes* on Pacific coast hosts differ from those described on Atlantic hosts by Coe (1902) and Humes (1942). Individual specimens of *C. carcinophila* on the Atlantic blue crab, *Callinectes sapidus*, are found encysted between the gill lamellae on non-ovigerous hosts. Hopkins (1947) found that these worms could move back and forth from gills to egg clutches through the summer, since *C. sapidus* no longer molts after maturity and produces several broods during the summer. *Carcinonemertes* can reach a length of more than 70 mm on blue crabs, compared to a maximum of about 10 mm on Pacific coast hosts, which brood only once a year.

Feeding by *C. errans* begins when female Dungeness crabs oviposit their broods during October and November. This host carries up to 2,500,000 eggs for a period of approximately 90 days. *C. errans* juveniles move into the egg clutch a day or two after host oviposition.

During its period in the host egg clutch *C. errans* differs from all other described *Carcinonemertes* in the lack of a sheath. Members of other species secrete a mucous sheath in which they live. Often males and females will be found together in these sheaths. *C. errans* remains free-crawling while on host eggs, secreting copious quantities of mucus, apparently for adhesion.

All measurable growth of *C. errans* occurs during its period in the host egg clutch. Wickham (1979b) found that the average size of worms within a population from a single host was correlated with the number of crab eggs eaten per worm on that host. Female worms are larger than male worms. However, the opacity of the epidermis in *C. errans* makes sex determination more difficult than in other local species which are more transparent.

Worm eggs hatch near the time of host eclosion and the worm larvae become planktonic. They remain so in the laboratory for at least one month. Thus it is unlikely that they reinfest the parental host as suggested for *C. carcinophila* by Humes (1942).

The density-dependent decline in fecundity found in laboratory-reared worms and the decrease in size of worm egg strings in field samples correlate with the decline in feeding and growth (Wickham, 1979b). This decline may reflect the action of some form of active intraspecific interference by *C. errans*. It occurs before food resources appear to be limiting, since in the test tube cultures crab egg mortality never exceeded 4%, even in tubes with eight worms.

Carcinonemertes errans is so far the only described species of *Carcinonemertes* restricted to a single host. *C. carcinophila* has been described from 26 host species in the Atlantic, Caribbean, and Mediterranean (Humes, 1942; Kirsteuer, 1966; Vivares, 1975; Norse, 1975). *C. epialti* has been described on nine hosts (Humes, 1942; Kuris, 1978; Roe, 1979) and *C. mitsukurii* from five hosts ranging from Japan to the Indo-Pacific and Hawaii (Humes, 1942). Wickham (1978) indicated that morphological characteristics in the sheaths of the other locally occurring *Carcinonemertes* spp. suggest that host specialization in this genus is more common than previously thought.

The biology of *C. errans* appears to adapt it specificially to its host. The fact that it was never found on alternate hosts such as *Cancer gracilis,* which shares the habitat of the Dungeness crab, suggests an ability to recognize its host. Its ability to transfer from the old exoskeleton during molting suggests a sophisticated behavioral repertoire, and the differential distribution on male and female exoskeletons shows an ability to discern the sex of the host.

Timing of development during the worm's trophic period appears to be an important adaptation in *C. errans,* especially when compared to worms on other hosts. *C. errans* matures and lays its eggs within about 2 weeks of the end of the host's brooding, after 65–70 days on the egg clutch. The time of development to worm hatching was not measured, but hatching was found to occur usually just prior to and during host hatching. This arrangement of timing would appear to allow worms the maximum amount of time for feeding on host eggs. Kuris (1978) and Roe (1979) found a similar synchronicity in the development of *C. epialti* on the host *H. oregonensis.*

Egg predators abound in nature (Orians and Janzen, 1974), but few specialize to the extent that *Carcinonemertes* does. The lifestyle of this genus shares features with parasitic castrators; however, *Carcinonemertes* is a true predator since it eats several distinct prey individuals during its life (Kuris, 1974).

The widespread occurrence of *Carcinonemertes* on numerous species of brachyuran crabs, many of great ecological and economic significance, gives these nemerteans an important role in benthic marine communities. This role is only beginning to be unraveled and the interaction of these worms with their hosts should provide stimulating material for a wide variety of studies on the adaptation of organisms to specialized environments.

This work is the result of research leading to the Ph.D. degree from University of California, Berkeley. I would like to thank Cadet Hand for continuing advice and guidance throughout this study. I would also like to thank Louis Botsford, Lynn Suer, Dennis Hedgecock, and Keith Nelson for criticism of this manuscript.

This work is the result of research sponsored by NOAA, Office of Sea Grant, Department of Commerce, under Grants No. NOAA 158-44121-R/A-19 and No. NOAA M01-189-R/F-52.

SUMMARY

1. Specimens of *Cancer magister* below 20 mm carapace width are not infested by *Carcinonemertes errans.* Worms infesting young-of-the-year crabs beyond this size would have been in the plankton for 8–9 months prior to host infestation.

2. Nemertean burden on crabs increases with the crab's time on the bottom, at least through the crab's early life. Worms move from the host's exuvium to its new exoskeleton upon host molting.

3. Juvenile specimens of *C. errans* were localized under the abdomen, near the copulatory appendages on male crabs. Juvenile worms were found in protected spots all over female crabs' exoskeletons.

4. Nemerteans migrate to the host egg clutch within a day or two of host ovipostion. They are peripherally distributed in the egg clutch through the early part of host brooding, but descend into the clutch to become more evenly distributed in the latter part of the brooding period.

5. All measurable growth occurs during *C. errans'* feeding period in the host egg clutch. Growth is inhibited as the density of worms per egg clutch increases.

6. *Carcinonemertes errans* matures approximately 60–70 days after host oviposition. Worms lay an average of 3.1 egg strings in the laboratory, each containing an average of 446 eggs. Fecundity per worm declines as worm density within the host egg clutch increases.

7. More than 99% of all specimens of *C. magister* are infested by *C. errans* in California waters. Numbers per host can range as high as 100,000.

8. Worms exhibit a contagious distribution among hosts, with the variance in density per host in excess of the mean density in all sample collections. The ratios of variance to mean increase as mean density increases.

LITERATURE CITED

BOTSFORD, L. W., AND D. E. WICKHAM, 1978. Behavior of age-specific, density-dependent models and the Northern California Dungeness crab fishery. *J. Fish. Res. Board Can.*, **35**: 833–843.

COE, W. R., 1902. The nemertean parasites of crabs. *Am. Nat.*, **36**: 431–450.

HOPKINS, S. H., 1947. The nemertean *Carcinonemertes* as an indicator of the spawning history of the host, *Callinectes sapidus*. *J. Parasitol.*, **33**: 146–150.

HUMES, A. G., 1942. The morphology, taxonomy and bionomics of the nemertean genus *Carcinonemertes. Ill. Biol. Monogr.*, **18**: 1–105.

KIRSTEUER, F., 1966. Uber *Carcinonemertes carcinophila* aus der Nordadria. *Zool. Anz.*, **176**: 205–212.

KURIS, A. M., 1971. *Population interactions between a shore crab and two symbionts.* Ph.D. thesis, University of California, Berkeley. 431 pp.

KURIS, A., 1974. Trophic interactions: Similarity of parasitic castrators and parasitoids. *Q. Rev. Biol.*, **49**: 129–148.

KURIS, A., 1978. Life cycle, distribution and abundance of *Carcinonemertes epialti,* a nemertean egg predator of shore crab, *Hemigrapsus oregonensis,* in relation to host size, reproduction and molt cycle. *Biol. Bull.*, **154**: 121–137.

NORSE, E. A., 1975. *The ecology of blue crabs, genus* Callinectes (*Brachyura: Portunidae*) *in the Caribbean.* Ph.D. thesis, University of Southern California. 259 pp.

ORIANS, G. H., AND D. H. JANZEN, 1974. Why are embryos so tasty? *Am. Nat.*, **108**: 581–592.

ROE, P., 1979. Aspects of the development of *Carcinonemertes epialti* in Elkhorn Slough, California. *Biol. Bull.*, **156**: 130–140.

VIVARES, P., 1975. Parasites de crustacés décapodes. *Ann. Parasit. Humane et Comparée,* **46** : 1–9.

WICKHAM, D. E., 1978. A new species of *Carcinonemertes* (Nemertea: Carcinonemertidae) with notes on the genus from the Pacific coast. *Proc. Biol. Soc. Wash.,* **91** : 197–202.

WICKHAM, D. E., 1979a. *The crab egg predator* Carcinonemertes errans *and population cycling and collapse in its host, the Dungeness crab.* Ph.D. thesis, University of California, Berkeley. 90 pp.

WICKHAM, D. E., 1979b. *Predation by the nemertean* Carcinonemertes errans *on eggs of the Dungeness crab,* Cancer magister. *Mar. Biol.,* **55** : 45–53.

Continued from Cover Two

include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by Biological Abstracts and Chemical Abstracts, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by Biological Abstracts (biosis List of Serials; most recent issue, 1976). Foreign authors, and others who are accustomed to use The World List of Scientific Periodicals, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. at £0.65 or $1.75) useful, since it sets out the World List abbreviations for most biological journals with notes of the USASI abbreviations where these differ. Chemical Abstracts publishes quarterly supplements of additional abbreviations. The following points of reference style for The Biological Bulletin differ from USASI (or modified World List) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in World List *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (not strictly as USASI usage, *i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.* not *J.Cell.Comp.Physiol.*)

E. We strongly recommend that more unusual words in journal titles be spelled out in full, rather than employing lengthy, peculiar "abbreviations" or new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation. Even in more familiar languages, *Z. Vererbungslehre* is preferred to *Z. VerebLehre* (World List) or *Z. Vererbungsl.* (USASI). *Accurate and complete communication of the reference is more important than minor savings in printing costs.*

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some Biological Abstracts listings).

H. Spell out *London, Tokyo, Paris, Edinburgh, Lisbon, etc.* where part of journal title.

I. Series letters *etc.* immediately before volume number.

J. A few well-known international journals in their preferred forms rather than World List or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond.; Science, N.Y.; Evolution, Lancaster, Pa.*)

5. ***Figures.*** Figures should be no larger than $8\frac{1}{2}$ by 11 inches. For review purposes, they should be submitted in triplicate, including three sets of photographic prints of all photomicrographs. The dimensions of the printed page, 5 by $7\frac{3}{8}$ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction. Explanatory matter should be included separately in legends as far as possible, although the axes should always be numbered and identified on the illustration itself. Figures should be prepared for reproduction as either line-cuts or halftones. Figures to be reproduced as line-cuts should be unmounted, glossy photographic reproductions or drawn in black ink on white paper, good quality tracing cloth or plastic, or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on board, and both designating numbers or letters and scale-bars should be affixed directly on the figures. We recommend that figures be about $1\frac{1}{2}$ times the linear dimensions of the final printing desired. *All* figures should be numbered in consecutive order, with no distinction between text- and plate-figures. The author's name should appear on the reverse side of all figures.

Reprints. Reprints may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

CONTENTS

ANNUAL REPORT OF THE MARINE BIOLOGICAL LABORATORY........ 1

ARNOLD, JOHN M., AND LOIS D. WILLIAMS-ARNOLD
Development of the ciliature pattern on the embryo of the squid, *Loligo pealei:* A scanning electron microscope study............. 102

BIGGER, CHARLES H.
Interspecific and intraspecific acrorhagial aggressive behavior among sea anemones: A recognition of self and not-self............ 117

HATCH, WALTER I.
The implication of carbonic anhydrase in the physiological mechanism of penetration of carbonate substrata by the marine burrowing sponge *Cliona celata* (Demospongiae)......................... 135

HILDRETH, JANE E., AND WILLIAM B. STICKLE
The effects of temperature and salinity on the osmotic composition of the southern oyster drill, *Thais haemastoma*................. 148

NELSON, KEITH, DENNIS HEDGECOCK, WILL BORGESON, ERIC JOHNSON, RICHARD DAGGETT, AND DIANE ARONSTEIN
Density-dependent growth inhibition in lobsters, *Homarus* (Decapoda, Nephropidae)................................ 162

SEIGFRIED, CLIFFORD A.
Seasonal abundance and distribution of *Crangon franciscorum* and *Palaemon macrodactylus* (Decapoda, Caridae) in the San Francisco Bay-Delta...................................... 177

SEIGFRIED, CLIFFORD A., AND MARK E. KOPACHE
Feeding of *Neomysis mercedis* (Holmes)..................... 193

STEEL, C. G. H.
Mechanisms of coordination between moulting and reproduction in terrestrial isopod Crustacea................................ 206

SUGIMOTO, KEIJI, AND HIROSHI WATANABE
Studies on reproduction in the compound ascidian, *Symplegma reptans:* Relationship between neural complex and reproduction... 219

WARNER, JON A., AND JAMES F. CASE
The zoogeography and dietary induction of bioluminescence in the midshipman fish, *Porichthys notatus*....................... 231

WICKHAM, DANIEL E.
Aspects of the life history of *Carcinonemertes errans* (Nemertea: Carcinonemertidae), an egg predator of the crab *Cancer magister*.. 247

Volume 159 Number 2

THE

BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

DANIEL L. ALKON, National Institutes of Health and Marine Biological Laboratory

FREDERICK B. BANG, Johns Hopkins University

EDWARD M. BERGER, Dartmouth College

STEPHEN C. BROWN, State University of New York at Albany

HARLYN O. HALVORSON, Brandeis University

J. B. JENNINGS, University of Leeds

MEREDITH L. JONES, Smithsonian Institution

GEORGE O. MACKIE, University of Victoria

JOEL L. ROSENBAUM, Yale University

HOWARD A. SCHNEIDERMAN, Monsanto Company

F. JOHN VERNBERG, University of South Carolina

E. O. WILSON, Harvard University

Managing Editor: CHARLES B. METZ, University of Miami

OCTOBER, 1980

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $10.00. Subscription per volume (three issues), $27.00 (this is $54.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and to Dr. Charles B. Metz, Institute For Molecular and Cellular Evolution, University of Miami, 521 Anastasia, Coral Gables, Florida 33134 during the remainder of the year. Assistant editor, Susan Schwartz.

Copyright © 1980, by the Marine Biological Laboratory

Second-class postage paid at Woods Hole, Mass., and additional mailing offices.
ISSN 0006-3185

INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except for a limited number of solicited review papers which may be accepted after formal refereeing), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts, including figures, should be submitted in triplicate. (Xerox copies of photographs are not acceptable for review purposes.) The original manuscript must be typed in double spacing (*including* figure legends, footnotes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. Manuscripts should be proofread carefully and errors corrected legibly in black ink. Pages should be numbered consecutively. Margins on all sides should be at least 1 inch (2.5 cm). Manuscripts should conform to the *Council of Biology Editors Style Manual*, 4th Edition (Council of Biology Editors, 1978). Unusual abbreviations should be kept to a minimum and should be spelled out on first reference as well as defined in a footnote on the title page. Manuscripts should be divided into the following components: Title page, Abstract (of no more than 200 words), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, Tables, and Figure Legends. In addition, authors should supply a list of words and phrases under which the article should be indexed.

2. ***Figures.*** Figures should be no larger than 8½ by 11 inches. The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

3. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the

Continued on Cover Three

Vol. 159, No. 2 October 1980

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

Reference: *Biol. Bull.*, **159**: 259–268. (October, 1980)

HEMOLYSINS AND HEMAGGLUTININS IN THE COELOMIC FLUID OF A POLYCHAETE ANNELID, *GLYCERA DIBRANCHIATA*

ROBERT S. ANDERSON

Sloan-Kettering Institute for Cancer Research, Donald S. Walker Laboratory,
145 Boston Post Road, Rye, New York 10580

The specificity and anamnestic characteristics of immune responses of annelids, particularly cellular reactions to integrementary grafts between oligochaetes, have been the subject of considerable study and controversy (*e.g.*, Duprat, 1967; Cooper, 1969; Hostetter and Cooper, 1972, 1973; Parry, 1978; Dales, 1978a, b). There is little doubt that the amoebocytic coelomocytes of annelids can differentiate between material of self and nonself origin; these cells are active in phagocytosis, encapsulation, and wound healing (Dales, 1978c), and may actively follow chemotactic gradients toward bacteria and foreign tissues (Marks *et al.*, 1979). Considerably less is known of the humoral immune factors present in the coelomic fluid of annelids, especially polychaetes, and the extent of cooperation between humoral and cellular components of the immune response is uncertain. Oligochaete coelomic fluid contains natural hemolytic activity (Chateaureynaud-Duprat and Izoard, 1973; Roch, 1979) suggested to play a role in graft rejection (Chateaureynaud-Duprat and Izoard, 1977a), and hemagglutinins that also possibly serve as humoral recognition factors in various foreign-body responses (Cooper *et al.*, 1974). This paper presents a description of naturally occurring hemolysins and hemagglutinins in the coelomic fluid of *Glycera*, and results of attempts to induce these factors by several experimental protocols. It is probable that these factors participate in polychaete immune mechanisms.

In most annelids there are two separate fluid-filled compartments, the coelom and the vascular system. However, in *Glycera* and a few other polychaetes, a separate blood system has disappeared (Dales, 1970). The coelomic fluid contains several easily identified cell types: erythrocytes, amoebocytes, and occasionally gametes. The cells will settle out, or can be removed by centrifugation, to leave the pale straw-colored supernatant fluid used in these studies. There is no evidence of immunoglobulins in this fluid, or in the coelomic fluid or hemolymph of

Copyright © 1980, by the Marine Biological Laboratory
Library of Congress Card No. A38–518
(ISSN 0006–3185)

any invertebrate. However, other factors with immunological properties are present.

Materials and Methods

Animals and collection of coelomic fluid

Specimens of *Glycera dibranchiata* were purchased from the Maine Bait Company, Newcastle, Maine. They were maintained in recirculated-water aquaria containing Instant Ocean artificial sea water at 11°–13° C. The worms usually remained burrowed in the calcareous gravel substrate, emerging occasionally to swim about near the bottom.

Coelomic fluid was collected in a plastic petri plate as it flowed from a small incision penetrating the integrement near the anterior end of the worm. Each worm yielded 0.5–1.0 ml of coelomic fluid. Samples were not pooled, except where otherwise indicated. Clotting was minimal, although some clumping of the amoebocytes was observed. Attempts to withdraw coelomic fluid by hypodermic syringe usually caused considerable lysis of *Glycera* red blood cells; this did not occur in fluid obtained as outlined above. Cells were removed from the fluid phase by centrifugation at 525 × *g* for 4 min at 4° C.

Hemolysin assay

Test mammalian erythrocytes (1% in Hanks' balanced salts solution, 0.5 ml) were added to an equal volume of coelomic fluid, or coelomic fluid serially diluted with Hanks' balanced salts solution (BSS). This mixture was incubated 60 min at 21° C. Two milliliter Hanks' BSS were added and the tubes immediately centrifuged for 3 min at 750 × *g*. The optical density (O.D.) of the supernatant was determined at 541 nm. A coelomic-fluid color-control tube was prepared by following the above procedure, but substituting Hanks' BSS for the test erythrocyte suspension. All values were corrected by subtracting the O.D. of the coelomic fluid color control. Test erythrocytes, following the above procedure, were incubated in distilled water, and the resultant O.D., representing 100% lysis, was measured. Using this value, the corrected experimental O.D. was converted to percent lysis. This basic procedure was modified in several ways as described in the "Results" section. For example, the incubation time was varied to determine reaction kinetics, the coelomic fluid serially diluted to assay hemolysin titer, EDTA added to determine requirement for divalent cations, different types of test erythrocytes were used, or coelomic fluid was obtained from animals previously injected with erythrocytes.

Hemagglutination assay

Hemagglutination assays were carried out in Cooke "U" well microtiter plates. The first well contained 100 μl coelomic fluid, 50 μl of which was then mixed with an equal volume of saline (0.9% NaCl) in the next well. In this way the coelomic fluid was serially diluted with a microdiluter through 10 wells. A control well contained saline only. Test erythrocytes (50 μl, 2% erythrocytes) were added to all wells and the microtiter plates covered and incubated for 60 min at 21° C. The hemagglutinin titers were read immediately after incubation.

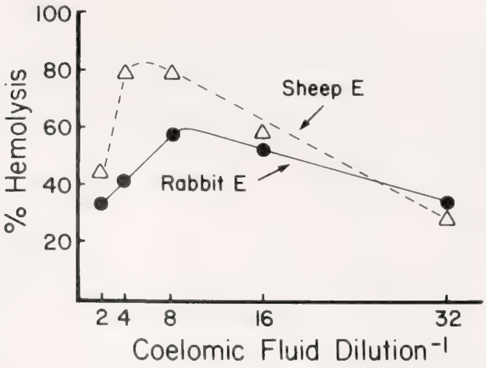


FIGURE 1. Effect of concentration on hemolytic activity of *Glycera* coelomic fluid. Erythrocytes (1%) were incubated with coelomic fluid for 60 min at 21°C. Each point is the mean of two separate determinations.

In certain cases, formalin-treated erythrocytes were used to measure hemagglutinin activity. These were prepared by exposure to 6% formaldehyde for 24–48 hr at 4°C. The cells were then washed five times and finally a 10% stock suspension in 0.15 M phosphate buffer (pH 7.0) was prepared. Aliquots of the stock solution were diluted to 2% for use in the hemagglutination assays.

Hemolysin and hemagglutinin induction

Hemolysin activity and hemagglutinin titer were determined in specimens of *Glycera* which had previously been injected with erythrocytes. The various injection schedules are described in the "Results" section. Each injection was intracoelomic and consisted of 0.1 ml of 10 or 50% erythrocyte suspension in sterile sea water. The injections had no effect on the behavior, morbidity, or mortality of the worms. At various intervals after injection, coelomic fluid was collected and hemolysin and hemagglutinin assays carried out as described above.

RESULTS

Hemolysis

Glycera. Coelomic fluid was shown to contain naturally-occurring hemolysin(s) against several mammalian erythrocytes (E). Maximal hemolysis was recorded at coelomic fluid dilutions of about 1/8 for rabbit E and 1/4–1/8 for sheep E. Sheep E were more susceptible than rabbit E to the lytic action of coelomic fluid at concentrations > 1/16 (Fig. 1). It was unusual to detect spectrophotometrically any E lysis at coelomic fluid dilutions greater than 1/128. The rate of sheep E hemolysis exceeded that of rabbit E (Fig. 2). The initial rate of reaction was rapid: In 1 hr about 90% of a standard suspension of sheep E was lysed by 1/8 coelomic fluid, while under identical conditions ~ 60% of a comparable suspension of rabbit E was lysed. Total hemolysis of the sheep E was reached by 2 hr; the percentage hemolysis of rabbit E increased very slowly to about 70% by 24 hr. Hemolysis assays were routinely carried out at room temperature (~ 21°C). Lowering the temperature of incubation to 4°C had no effect on hemolytic activity: When the study presented in Figure 2 was repeated at 4°C,

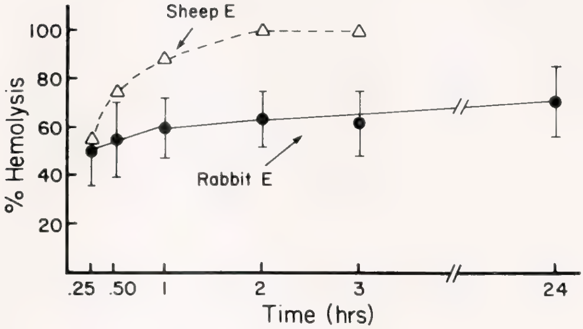


FIGURE 2. Kinetics of hemolytic activity of *Glycera* coelomic fluid. Erthyrocytes (1%) were incubated with 1/8 diluted coelomic fluid at 21°C. Means ± standard deviations (vertical lines) (N = 5) are given for rabbit erythrocytes; results of one representative experiment using sheep erythrocytes are included for comparative purposes.

none of the mean percent hemolysis values were statistically different from those recorded at 21°C. Attempts to preserve hemolytic activity at –80°C after quick freezing with ethanol and dry ice were unsuccessful; apparently biological activity was lost during freezing and thawing. The hemolysin was inactivated by heating to 56°C for 30 min. If the coelomic fluid was treated with > 1 mM EDTA (ethylenediamine tetraacetic acid, disodium salt) at neutral pH, its hemolytic activity was markedly inhibited (Fig. 3).

The hemolytic potential of coelomic fluid was considerably reduced by adsorption with erythrocytes (Table I). Adsorption with rabbit E reduced subsequent lysis of rabbit E or sheep E to a comparable extent. Sheep E were more efficient in adsorbing anti-rabbit and anti-sheep hemolysins than rabbit E. Hemolysis of sheep E was more inhibited by adsorption with autologous E than was hemolysis of rabbit E, following each of the first two adsorptions.

Hemolysin activity was determined daily for three days after a single injection of a 50% suspension of sheep or rabbit E (Table II). Injection with rabbit E produced no significant effect on anti-sheep hemolysin activity, but caused a reduction in the lysis of rabbit E, which lasted for the course of the experiment. Injection of sheep E caused a persistent reduction of both anti-sheep and anti-rabbit

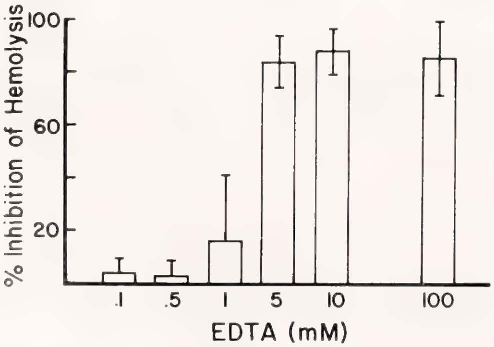


FIGURE 3. Inhibition of *Glycera* anti-rabbit-erythrocyte lysin by EDTA. Rabbit red blood cells (1%) were incubated with EDTA-treated coelomic fluid (1/8 dilution) for 60 min. Mean % inhibition ± standard deviation (vertical lines) (N = 5) are given for each EDTA concentration.

TABLE I

Effect of adsorption with erythrocytes on Glycera *hemolysin. Each adsorption consisted of 15 min incubation at 1°C of 1 vol freshly packed erythrocytes resuspended in 2 vol coelomic fluid. Means and standard deviations are given for the first two adsorptions ($N = 3$, for each value); three adsorptions were carried out on only one pool of coelomic fluid.*

Coelomic fluid adsorbed with:	Hemolysis (% of unadsorbed coelomic fluid) vs. rabbit erythrocytes	Hemolysis (% of unadsorbed coelomic fluid) vs. sheep erythrocytes
Rabbit erythrocytes		
1x	59.5 ± 20.6	58.7 ± 0.6
2x	40.4 ± 13.6	30.3 ± 9.9
3x	19.7	17.1
Sheep erythrocytes		
1x	44.2 ± 17.3	29.1 ± 6.5
2x	13.4 ± 10.8	5.1 ± 1.6
3x	3.9	3.0

hemolysin. In all cases following a single injection of E, there was a gradual tendency for the decreased levels of hemolysins to return to control levels with time. The hemolysin values from uninjected animals were valid controls for this study because the mean hemolytic activity in uninjected animals was not significantly different from that of *Glycera* injected with sterile sea water.

In three studies comparable to that reported in Table II, hemolytic activity was followed after a single 0.1 ml injection of a 10% suspension of sheep E or

TABLE II

Hemolysin activity (% hemolysis) in Glycera *coelomic fluid after injection of mammalian erythrocytes (E), 0.1 ml, 50% E in sterile sea saline. Mean ± SD (N). Probability* (P) *values from two-tailed* t *test comparing % hemolysis mediated by coelomic fluid of injected animals to controls.*

Indicator E	Percent hemolysis by coelomic fluid			
	Uninjected	Time after intracoelomic injection of rabbit E		
		24 hr	48 hr	72 hr
Sheep E	73.9 ± 16.7 (25)	60.2 ± 28.3 (6) N.S.	64.7 ± 22.4 (6) N.S.	71.0 ± 27.2 (6) N.S.
Rabbit E	58.5 ± 8.7 (25)	32.3 ± 19.3 (6) $P < 0.01$	30.3 ± 18.9 (6) $P < 0.01$	36.0 ± 15.0 (6) $P < 0.01$
	Uninjected	Time after intracoelomic injection of sheep E		
		24 hr	48 hr	72 hr
Sheep E	73.9 ± 16.7 (25)	37.8 ± 22.7 (6) $P < 0.01$	41.4 ± 11.8 (6) $P < 0.01$	55.4 ± 15.4 (6) $P < 0.05$
Rabbit E	58.5 ± 8.7 (25)	21.0 ± 20.5 (6) $P < 0.01$	25.6 ± 12.0 (6) $P < 0.01$	26.9 ± 10.4 (6) $P < 0.01$

TABLE III

Effect of multiple intracoelomic injections of mammalian erythrocytes (0.1 ml, 50% E in sterile sea saline) on hemolysin activity in Glycera *coelomic fluid. Mean ± SD (N). Two-tailed* t *test comparing % hemolysis mediated by coelomic fluid of injected animals to controls; for all comparisons the value of* t *is at the 0.01 level of significance. Regimen I:* Glycera *injected at 0 hr, 24 hr, and coelomic fluid sampled at 48 hr. Regimen II:* Glycera *injected at 0 hr, 24 hr, 72 hr, and coelomic fluid sampled at 96 hr.*

Indicator E	Percent hemolysis by coelomic fluid		
	Uninjected	Intracoelomic injection of rabbit E	
		Regimen I	Regimen II
Sheep E	73.9 ± 16.7 (25)	52.9 ± 12.9 (6)	49.1 ± 19.3 (6)
Rabbit E	58.5 ± 8.7 (25)	18.7 ± 14.3 (6)	21.5 ± 15.6 (6)
	Uninjected	Intracoelomic injection of sheep E	
		Regimen I	Regimen II
Sheep E	73.9 ± 16.7 (25)	33.1 ± 20.1 (6)	28.4 ± 25.9 (6)
Rabbit E	58.5 ± 8.7 (25)	12.5 ± 10.0 (6)	18.5 ± 17.2 (6)

rabbit E. The previously observed decreases in hemolytic activities after injection of 0.1 ml 50% E were not seen. Hemolysin activity in 10%-E-injected animals was not significantly different from controls.

Table III shows the effect of multiple intracoelomic injections of E on *Glycera* hemolysin. In one set of experiments, E was injected at 0 and 24 hr, and coelomic fluid sampled at 48 hr. In another set, E was injected at 9, 24, and 72 hr, and coelomic fluid was sampled at 96 hr. Repeated E injections, regardless of which regimen was used, reduced the hemolysin activity more than a single E injection. Injecting rabbit E according to these schedules significantly inhibited anti-sheep hemolysin, an effect not produced by a single injection.

Hemagglutinin

Glycera coelomic fluid contained hemagglutinating factor(s) active against both rabbit and sheep erythrocytes (Table IV). Rabbit E were agglutinated at significantly ($P < .005$) lower concentration of coelomic fluid than were sheep E.

TABLE IV

Hemagglutinin activity (titer) in Glycera *coelomic fluid against formaldehyde-treated erythrocytes (f E) and untreated erythrocytes (E). Titer =* Log_2^{-1} $\bar{x}$ *lowest coelomic fluid concentration giving visible hemagglutination ± SD (N).*

Indicator E	Hemagglutinin titer
Sheep E	2.9 ± 0.3 (10)
f Sheep E	6.9 ± 1.2 (20)
Rabbit E	4.6 ± 0.7 (10)
f Rabbit E	4.4 ± 0.6 (20)

Erythrocytes pretreated with formaldehyde were more strongly agglutinated at any given dilution of coelomic fluid than untreated E. Furthermore, anti-formalin-treated sheep-E hemagglutinin titers were significantly ($P < 0.005$) higher than those of untreated sheep E. Formalin treatment of rabbit E did not increase hemagglutinin titers.

The hemagglutinin did not appear to require divalent cations for activity; EDTA at 50 mM or less had negligible effect on its activity. Freezing and thawing had no effect on the hemagglutinating activity of coelomic fluid. However, samples heated to 56°C for 30 min had only 5–10% of the hemagglutinating capacity of untreated coelomic fluid.

Coelomic fluid was adsorbed three times with test Es, as described in Table I. Adsorption with either sheep E or rabbit E caused a reduction of both anti-sheep-E and anti-rabbit-E hemagglutinin; neither E type was more effective than the other as a hemagglutinin-adsorbing agent.

Attempts to alter hemagglutinin activity by prior intracoelomic injection of mammalian erythrocytes were ineffective. *Glycera* were injected with either sheep E or rabbit E; hemagglutinin titers were determined according to the schedules described previously for the hemolysin assays. Animals were injected with 0.1 ml suspension of either 10% or 50% E; neither anti-sheep-E nor anti-rabbit-E hemagglutinin titers showed significant change over a 72-hr period. Typical data comparing control values to 24-hr post-E injection titers are given in Table V. Multiple injections of 0.1 ml of 50% E suspension, administered according to the protocols given in the hemolysin section, also produced no increase in hemagglutinin titer.

DISCUSSION

The coelomic fluid of *Glycera* contained naturally occurring lytic and agglutinating factors active against both sheep and rabbit erythrocytes. Based on their activity in the presence of EDTA, *Glycera* hemolysin required Ca^{2+} or Mg^{2+} for activity, whereas the hemagglutinin did not. Similar findings had been reported for the hemolysin and hemagglutinin of *Lumbricus terrestris* (Cooper *et al.*, 1974) and *Amphitrite ornata* (Garte and Russell, 1976), although Roch (1979) reported no EDTA inhibition of *Eisenia fetida* hemolysin. Many invertebrate hemagglutinins require Ca^{2+} for biological activity; for example, those from *Limulus* (Marchalonis and Edelman, 1968), lobsters (Hall and Rowlands, 1974), oysters (Acton *et al.*, 1969), and tunicates (Anderson and Good, 1975). However, other invertebrate lectins do not show a dependency on divalent cations; oyster hemagglutinin is active against sheep E and rabbit E in the absence of exogenous Ca^{2+} (McDade and Tripp, 1967), and insectan hemagglutinin is active in the presence

TABLE V

Hemagglutinin titer in Glycera *coelomic fluid 24 hr after an injection of erythrocytes (0.1 ml, 10%, E suspension). Titer = Log_2^{-1} $\bar{x}$ lowest coelomic fluid concentration giving visible agglutination of formaldehyde-treated erythrocytes (f E) ± SD (N).*

Indicator E	Uninjected	Rabbit E injected	Sheep E injected
f Sheep E	7.2 ± 0.9 (10)	7.5 ± 1.1 (10)	7.3 ± 0.9 (10)
f Rabbit E	4.7 ± 0.8 (10)	4.7 ± 2.1 (10)	4.4 ± 0.8 (10)

of EDTA (Anderson *et al.*, 1972). In lower vertebrates including sharks, rays, and paddlefish, and in higher vertebrates such as guinea pigs and man, agglutinins are not inhibited by EDTA; however, hemolysins are EDTA-sensitive (Gewurz *et al.*, 1966).

Both *Glycera* hemolysin and hemagglutinin were heat-inactivated by holding at 56°C for 30–60 min, as was the case for these humoral factors in oligochaetes (Cooper *et al.*, 1974; Roch, 1979). Garte and Russell (1976) report that a polychaete hemagglutinin, amphitritin, is active at temperatures below 85°C. Heat stability above ~ 70°C is uncommon for invertebrate lectins or hemolysins. Incubation of E with *Glycera* hemolysin at temperatures of 4°–30° had little effect on the rate or extent of lysis. Sipunculid worm coelomic fluid also contains a hemolysin that has unaltered activity when incubated with E over a 0°–25° temperature range (Weinheimer *et al.*, 1970). In this regard, it is interesting that the activity of hemolysins of poikilothermic vertebrates is increased at lower temperatures (Cushing, 1945; Gewurz *et al.*, 1966). Considerable activity of *Glycera* hemolysin is lost after freezing and thawing, a reaction not typical of hemolysins from other invertebrates such as *Mercenaria mercenaria* (Anderson, unpublished observation), the spiny lobster (Weinheimer *et al.*, 1969), and the earthworm (Roch, 1979). The hemagglutinin of *Glycera* does not lose activity after storage at − 80°C; this is typical of most invertebrate lectins (Pauley, 1974).

Since both hemolysins and agglutinins are postulated to play a role in the immune reactions of invertebrates, their target cell specificity should be considered. Based on our observations, both factors could react with E from several mammalian species: however, the intensity of the reaction against a particular type of indicator cell was different from that against another. Also, the hemolysin adsorption studies showed that, although there was considerable cross-reactivity, there was some evidence of specificity. While adsorption with either sheep E or rabbit E reduced hemolysin activity against both kinds of E, adsorption with sheep E reduced anti-sheep E lysin more than anti-rabbit E lysin. A similar directed effect on *Glycera* lysins was not seen after adsorption with rabbit E. Adsorption of coelomic fluid with either E type reduced agglutination of both equally.

It is not known if *Glycera* hemagglutinins and hemolysins are single entities with rather broad specificities or if they exist in multiple forms with more limited specificities. Current data suggest that both of these factors in other annelids may exist in multiple forms. Garte and Russell (1976) isolated and purified a hemagglutinin from the polychaete *Amphitrite ornata.* Sephadex G-100 fractionation yielded three active fractions with molecular weight of 30,000, 54,000, and 100,000. These fractions showed parallel specificity toward certain E, but different patterns of agglutination for other E types. The 30,000 dalton glycoprotein fraction (Amphitritin) probably was a subunit of the higher molecular weight agglutinin. Earthworm hemolysin was shown by the use of isoelectric focusing to be composed of four lipoprotein isoforms (Roch, 1979). Each isoform had natural lytic activity against sheep erythrocytes. One isoform was found in all *Eisenia fetida* studied. The other isoforms were variably present. It was suggested that the natural hemolysin of *Eisenia* was induced and played a role during second set graft rejection (Chateaureynaud-Duprat and Izoard, 1977a). However, Roch (1979) found no qualitative difference in the pattern of hemolytic proteins (isoforms) in grafted animals; the patterns remained stable regardless of grafting, wounding, starvation, or age of the worms.

It has been reported that hemolysins and hemagglutinins may be induced in terrestrial annelids by grafting or by the injection of erythrocytes (Cooper *et al.*, 1974; Chateaureynaud-Duprat and Izoard, 1977b). We were unable to induce in *Glycera* either hemolysins or hemagglutinins by either single or multiple intracoelomic injections of erythrocytes. Cooper *et al.* (1974) injected *Lumbricus* with 0.1 ml of a 10% E suspension; 24 hr later, lysis and agglutination of both sheep and rabbit E were enhanced. Chateaureynaud-Duprat and Izoard (1977b) reported that *Lumbricus* coelomic fluid had no agglutinating or lysing activity until 3–4 days after the injection of sheep E. None of our procedures involving single or multiple injections of 0.1 ml of 10 or 50% mammalian E caused any significant change in *Glycera* hemagglutinin titers over a 72–96 hr period of observation. No induction of hemolytic activity was achieved by our protocols; in most cases, hemolysin activity was significantly decreased following E injection. This reduction was more pronounced in the coelomic fluid of those worms that received multiple E injections, suggesting that the hemolysin was simply removed from circulation by adsorption to the membranes of the injected erythrocytes.

Studies of graft rejection and other manifestations of immunological memory among polychaetes have not been undertaken. However, our studies suggest that the naturally occurring hemolysins and hemagglutinins of *Glycera* are not inducible. Therefore, one would not predict that these humoral factors would play a significant role in anamnestic immune reactions. This is not to exclude the possibility that the factors may have an important function in a more primitive immunological recognition system.

This work was supported in part by grant OCE-7723443 from the National Science Foundation, NCI Core Grant CA 08748, and a grant from the Whitehall Foundation. The author wishes to acknowledge the excellent technical assistance of J. D. Pearlman.

SUMMARY

1. Naturally occurring hemolysins and hemagglutinins active against erythrocytes from two mammalian species were found in the coelomic fluid of the polychaete *Glycera dibranchiata*.

2. The hemolysin required divalent cations and was inactivated by freezing and thawing; the hemagglutinin retained activity after freezing and was active in the presence of EDTA. Both factors were thermolabile above 56°C.

3. The rate of reaction and degree of intensity of lysis or agglutination was different for each type of test erythrocyte. These differences were found in all individuals studied, and were considered to be characteristic of the species.

4. Both hemolysin and hemagglutinin were readily adsorbed from coelomic fluid by the addition of erythrocytes. Adsorption with a particular type of erythrocyte resulted not only in reduced lytic and agglutinating activity against that cell type, but also in reduced activity against red blood cells from other species.

5. The hemolysins and hemagglutinins of *Glycera* were not induced by single or multiple intracoelomic injections of erythrocytes. These experimental treatments had no significant effect on hemagglutinin titers, and usually caused a marked reduction in hemolysin activity. The consequence of the apparent lack of inducible humoral factors on possible anamnestic immune mechanisms, such as graft recognition and destruction, has yet to be evaluated.

LITERATURE CITED

Acton, R. T., J. C. Bennett, E. E. Evans, and R. E. Schrohenloher, 1969. Physical and chemical characterization of an oyster hemagglutinin. *J. Biol. Chem.*, **244**: 4128–4135.

Anderson, R. S., N. K. B. Day, and R. A. Good, 1972. Specific hemagglutinin and a modulator of complement in cockroach hemolymph. *Infect. Immun.*, **5**: 55–59.

Anderson, R. S., and R. A. Good, 1975. Naturally-occurring hemagglutinin in a tunicate *Halocynthia pyriformis*. *Biol. Bull.*, **148**: 357–369.

Chateaureynaud-Duprat, P., and F. Izoard, 1973. Etude des mécanismes de défense chez *Lumbricus terrestris*. *C. R. Acad. Sci. Paris*, **276**: 2859–2862.

Chateaureynaud-Duprat, P., and F. Izoard, 1977a. Etude comparée *in vitro* des réactions de defense antigreffe chez 2 genres de Lombriciens: *Eisenia* et *Lumbricus*. *C. R. Acad. Sci. Paris, Série D*, **284**: 2581–2584.

Chateaureynaud-Duprat, P., and F. Izoard, 1977b. Compared study of immunity between two genera of Lumbricians: *Eisenia* and *Lumbricus*. Pp. 33–40 in J. B. Solomon and J. D. Horton, Eds., *Developmental Immunobiology*. Elsevier/North-Holland Biomedical Press, Amsterdam.

Cooper, E. L., 1969. Specific tissue graft rejection in earthworms. *Science*, **166**: 1414–1415.

Cooper, E. L., C. A. E. Lemmi, and T. C. Moore, 1974. Agglutinins and cellular immunity in earthworms. *Ann. N. Y. Acad. Sci.*, **234**: 34–49.

Cushing, J. E., Jr., 1945. A comparative study of complement. I. The specific inactivation of the components. *J. Immunol.*, **50**: 61–74.

Dales, R. P., 1970. *Annelids*. Hutchinson & Co., Ltd., London. 200 pp.

Dales, R. P., 1978a. The basis of graft rejection in the earthworms *Lumbricus terrestris* and *Eisenia foetida*. *J. Invertebr. Pathol.*, **32**: 264–277.

Dales, R. P., 1978b. Second-set graft rejections: Do they occur in invertebrates? Pp. 203–220 in A. S. G. Curtis, Ed., *Cell-Cell Recognition*. Society for Experimental Biology Symposium No. 32. Cambridge University Press, England.

Dales, R. P., 1978c. Defense mechanisms. Pp. 479–507 in P. J. Mill, Ed., *Physiology of Annelids*. Academic Press, London.

Duprat, P., 1967. Etude de la prise et du maintien d'un greffon de paroi du corps chez le lombricien *Eisenia foetida typica*. *Ann. Inst. Pasteur (Paris)*. **113**: 867–881.

Garte, S. J., and C. S. Russell, 1976. Isolation and characterization of a hemagglutinin from *Amphitrite ornata*, a polychaetous annelid. *Biochim. Biophys. Acta*, **439**: 368–369.

Gewurz, H., J. Finstad, L. H. Muschel, and R. A. Good, 1966. Phylogenetic inquiry into the origins of the complement system. Pp. 105–116 in R. T. Smith, P. A. Miescher, and R. A. Good, Eds., *Phylogeny of Immunity*. University of Florida Press, Gainesville.

Hall, J. L., and D. T. Rowlands, Jr., 1974. Heterogeneity of lobster agglutinins. I. Purification and physicochemical characterization. *Biochemistry*, **13**: 821–827.

Hostetter, R. K., and E. L. Cooper, 1972. Coelomocytes as effector cells in earthworm immunity. *Immunol. Commun.*, **1**: 155–183.

Hostetter, R. K., and E. L. Cooper, 1973. Cellular anamnesis in earthworms. *Cell. Immunol.*, **9**: 384–392.

McDade, J. E., and M. R. Tripp, 1967. Mechanism of agglutination of red blood cells by oyster hemolymph. *J. Invertebr. Pathol.*, **9**: 523–530.

Marchalonis, J. J., and G. M. Edelman, 1968. Isolation and characterization of a hemagglutinin from *Limulus polyphemus*. *J. Mol. Biol.*, **32**: 453–465.

Marks, D. H., E. A. Stein, and E. L. Cooper, 1979. Chemotactic attraction of *Lumbricus terrestris* coelomocytes to foreign tissue. *Dev. Compar. Immunol.*, **3**: 277–285.

Parry, M. J., 1978. Survival of body wall autografts, allografts, and xenografts in the earthworm *Eisenia foetida*. *J. Invertebr. Pathol.*, **31**: 383–388.

Pauley, G. B., 1974. Physicochemical properties of natural agglutinins of some mollusks and crustaceans. *Ann. N. Y. Acad. Sci.*, **234**: 145–158.

Roch, P., 1979. Protein analysis of earthworm coelomic fluid: 1) Polymorphic system of the natural hemolysin of *Eisenia foetida andrei*. *Dev. Compar. Immunol.*, **3**: 599–608.

Weinheimer, P. F., R. T. Acton, J. E. Cushing, and E. E. Evans, 1970. Reactions of sipunculid coelomic fluid with erythrocytes. *Life Sci.*, **9**: 145–152.

Weinheimer, P. F., E. E. Evans, R. M. Stroud, R. T. Acton, and B. Painter, 1969. Comparative immunology: Natural hemolytic system of the spiny lobster, *Panulirus argus*. *Proc. Soc. Exp. Biol. Med.*, **130**: 322–326.

Reference: *Biol. Bull.*, **159**: 269–279. (October, 1980)

ALARM RESPONSE OF THE INTERTIDAL SNAIL *LITTORINA LITTOREA* (L.) TO PREDATION BY THE CRAB *CARCINUS MAENAS* (L.)

ROBIN P. HADLOCK[1]

Department of Biology, Bowdoin College, Brunswick, Maine, 04011, and
Department of Zoology, University of Rhode Island,
Kingston, Rhode Island 02880

Many aquatic organisms rely on chemical senses to detect predators. Often avoidance behavior is elicited by distance or contact chemoreception of predator "odor" or "taste" (Mackie and Grant, 1974). Some species, however, have evolved alarm or escape responses to juices from the injured tissues of crushed conspecifics; these behaviors are found in minnows (von Frisch, 1938), amphibian tadpoles (Kulzer, 1954), sea urchins (Snyder and Snyder, 1970), sea anemones (Howe and Sheikh, 1975) and gastropod molluscs (Kempendorff, 1942; Snyder, 1967; Snyder and Snyder, 1971; Atema and Burd, 1975; Atema and Stenzler, 1977; Stenzler and Atema, 1977).

Snyder found in laboratory studies that 19 of 30 snail species tested respond to conspecific juice. He suggested that, in general, alarm reactions are responses to predation. Predators were tested for their ability to crush snails and elicit alarm responses in the laboratory. However, until Ashkenas and Atema (1978) reported that burrowing *Ilyanassa obsoleta* are rarely attacked by *Carcinus maenas* in the laboratory, no studies had tested whether responding with alarm behavior helps an individual snail avoid being eaten. Direct field observations of predation, which could support the antipredator hypothesis, have been lacking.

The present study was undertaken to test this antipredator interpretation. This paper describes the alarm response of *Littorina littorea* and field and laboratory observations of *Carcinus* predation on *L. littorea* and presents results of studies testing the utility of alarm behavior in preventing crab predation.

MATERIALS AND METHODS

Alarm behavior of Littorina littorea

Field experiments on the alarm repsonse of *L. littorea* were performed in tide pools of the rocky intertidal mid- and high zones at Bailey Island, Georgetown, and Harpswell, Maine. Snails found in small pools (< 25 cm deep, < 0.75 m^2 area) were tested in order to present snails with a high concentration of snail juice in tide-pool water. Snails responded to crushed conspecifics by moving to sites in the pool where they were less visible to a human observer. This response was measured by placing an octagonal grid (60 cm diameter, suspended from a circular plastic frame) over the tide-pool surface. Each trial lasted 60 min and consisted of a 30 min control period (min 0–min 30) followed by the experimental period (min 30–min 60). At the beginning of the control period (min 0), two intact snails were dropped into the center of the grid area. At min 30 (beginning of experimental period) two crushed snails were dropped into the center of the grid area (N = 6 trials). The locations of snails visible under the grid were recorded at 10-min intervals for the full 60 min of each trial (after Atema and Burd, 1975).

---

[1] Present address: Department of Biology, Osborn Memorial Laboratories, Yale University, New Haven, Connecticut 06520.

Wet weight of snail tissue added to tide pools was determined by shell length–wet tissue weight regression. Mean wet tissue weight of intact snails added to pools was 0.68 ± 0.07 g, N = 6 trials (in this and subsequent sections, values are reported as means ± one standard error). Mean wet weight of crushed snail tissue added was 0.48 ± 0.20 g, N = 6 trials. Fifty-nine ± 24.1 (range 17–122) snails were followed per trial.

A second experimental series was designed to test for responses to chemical stimulation by the snail juice alone. At min 0 (beginning of control period) 6.25 ± 0.75 ml sea water was substituted for the intact snails of the first experimental series and at 30 min (beginning of experimental period) 5.45 ± 0.75 ml of filtered snail juice was substituted for the crushed snails (N = 4 trials). Snail juice was prepared at poolside just prior to each trial by crushing two individuals of *L. littorea* of known shell length (distance from apex to base of aperture) in a dish, adding sea water from another pool, and filtering the mixture through Whatman #1 filter paper into a 50 ml filtration flask. Both sea-water control and snail juice were released from a pipette into the center of the grid area; again, each snail juice test followed a control trial. Separate pipettes were used to avoid contamination between control and test stimuli. The concentration of snail juice added was estimated by first determining the wet weight of crushed tissue from shell length–wet tissue weight regression. The approximate concentration added was 0.12 g/ml of snail tissue in sea water before filtration and release into a tide pool. In each trial 66.5 ± 33.3 snails (range 35–106) were followed.

In two additional blank trials the experiment was conducted in the same way but the test stimulus at min 30 was omitted. In each trial 52 ± 36.9 snails (range 3–101) were followed.

To examine the rate of crawl of individual snails, in one Bailey Island pool 11 snails were marked individually with Pla enamel (Testors Corp., Rockford, Il.). Positions of six individuals were recorded at 10-min intervals during a 30-min control (sea water) and a 30-min experimental test (filtered snail juice) period. This pool was tested on April 14 and May 3, but not every snail marked was present for both tests. The movements of these individuals were also recorded during a 50-min blank trial on April 22 during which sea water, but not test stimulus, was added.

Predation by Carcinus maenas

To test the effectiveness of the *L. littorea* alarm response in preventing crab predation, the times required for crabs to find snails in "sheltered" and "exposed" sites were compared in the laboratory. Also, the time required for snails to hide was compared to the duration of the "consume phase" of crab feeding behavior (Fig. 2).

The first comparison was simplified by using only one type of sheltered site chosen by snails in the field: a rock crevice. Two round glass bowls (each 20 cm diameter, 6.5 cm deep) filled with sea water to a depth of 6.0 cm were used to simulate tide-pool habitat. The bottoms of these bowls were lined with several flat rocks. Crabs were tested with "exposed" snails by placing snails in the center of a rock surface at one end of the bowl. In trials with "sheltered" snails, snails were placed in the approximately 2.0-cm-deep crevices formed between rocks.

Crabs used in these experiments were collected by commercial fishermen in Rhode Island, held in a damp refrigerated room for 3 days, and then transferred to two large (20- and 45-gal) aquaria in a recirculating sea-water system until experiments began 4 days later. Crabs were not fed during this time. Individuals of *L. littorea* were collected in Narragansett, Rhode Island, on the first day of the experiment and held in a damp glass bowl thereafter.

Crabs were placed in the bowls, allowed to acclimate for 10–30 min, then removed for 2–5 sec while a snail was positioned in the bowl. Both pools were used in "sheltered" and "exposed" trials. Between trials, pools were rinsed with hot water and refilled with fresh sea water. Distances between crab and snail were the same (approximately 16 cm) at the start of all 12 trials. The experiment took place in a dark room with the pools lit by a microscope illuminator. Crabs were observed for 20 min or until they had picked up a snail and moved it to the "attack" position in front of the mouthparts.

In a second experiment the time required for crabs to injure and consume snails was estimated using the same glass bowls. The goal of this test was to determine how long a crab takes to consume one snail before searching for the next, since this is the period of time available to intact conspecifics to find shelter. The "consume phase" of the predation sequence begins with first injury to the snail body and release of snail juice to the surrounding water. The exact time of first injury was difficult to determine, so this moment was standardized by equating it with a behavior involving sure injury, the "pull from mouthparts" (see below). The end of the "consume phase" was marked by completion of all feeding behavior. Each crab was placed with a small (< 9.0 mm shell length), medium-sized ($\geqslant 9.0$, $\leqslant 18.0$ mm), and large (> 18.0 mm) snail (Underwood, 1973) and observed until all the snails in the bowl were consumed or the crab showed no searching behavior for 10 min after consuming a snail.

Field observations of *Carcinus-Littorina* interactions took place in tide pools at Appledore Island, Maine. Feeding crabs were found at night by sweeping the red beam of a 9 V lantern (lens covered with a #2423 red plexiglas disc) over pool bottoms. Crabs were also observed feeding along the stony bottom of a cove at high tide during the day.

RESULTS

Snail alarm behavior

The proportion of snails visible in the grid area decreased significantly in the 10 min period following addition of crushed snail ($P \leqslant 0.05$) or snail juice ($P \leqslant 0.025$) relative to changes in the proportion visible 10 min after introduction of intact snails or sea water (angular transformation of proportions; analysis of variance for paired data; Sokal and Rohlf, 1969). Snails tested in these trials hid by crawling into crevices, under fronds of macroalgae, or under rocks. In one pool, snails grazing at the tips of *Chondrus* blades moved down among the blades toward the holdfast. There was no significant change in the proportion of snails visible during the same intervals of blank trials (Fig. 1).

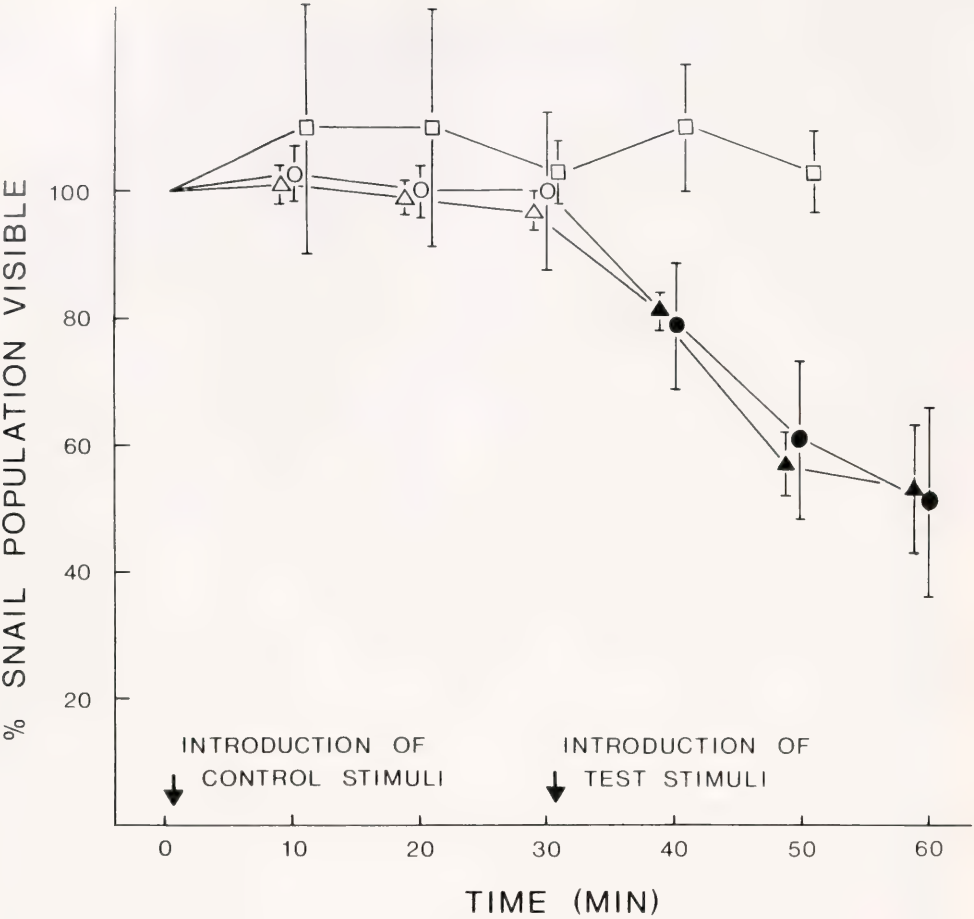


FIGURE 1. Percent of *L. littorea* populations visible following introduction of control and test stimuli, relative to percent visible at min 0. Stimuli introduced: open circle = intact *L. littorea* control; closed circle = crushed *L. littorea* (N = 6 trials). Open triangle = sea water control; closed triangle = crushed *L. littorea* juice (N = 4 trials). Open box indicates blank trials: intact snail or sea water was added at min 0 but no test stimulus was introduced (N = 2 trials). Symbols and bars represent means ± 1 standard error.

In general, snail activity in tide pools increased after addition of crushed snail or snail juice. Individuals in one pool increased rates of locomotion significantly, from 0.32 ± 0.07 cm/min (N = 6) in the 20-min interval preceding addition of snail juice to 1.40 ± 0.31 cm/min (N = 6) in the 20-min period following addition of juice ($P \leqslant 0.03$; Wilcoxon's signed-ranks test). In applying this statistical test, it was assumed that snails responded independently, although no experimental test for independent responses was conducted. There was no significant change in crawling velocity during the same periods of the blank trial.

Crab feeding behavior

Feeding crabs were observed in the laboratory and in the field. Detailed description of feeding behavior was based on laboratory observations.

Search phase. Feeding behavior begins when the crab detects, apparently by olfaction, the presence of nearby snails. First the antennule flicking rate increases (antennule beat, Fig. 2) and antennule position changes from primarily vertical to pointing at different angles from the carapace (antennule point, Fig. 2). The third maxillipeds then begin to sway from side to side and one may be wiped over the other several times. This may last for several minutes until the crab begins to move forward. The advance is accompanied by chelae and walking-leg raking, in which the chelae and walking legs are extended from the carapace and

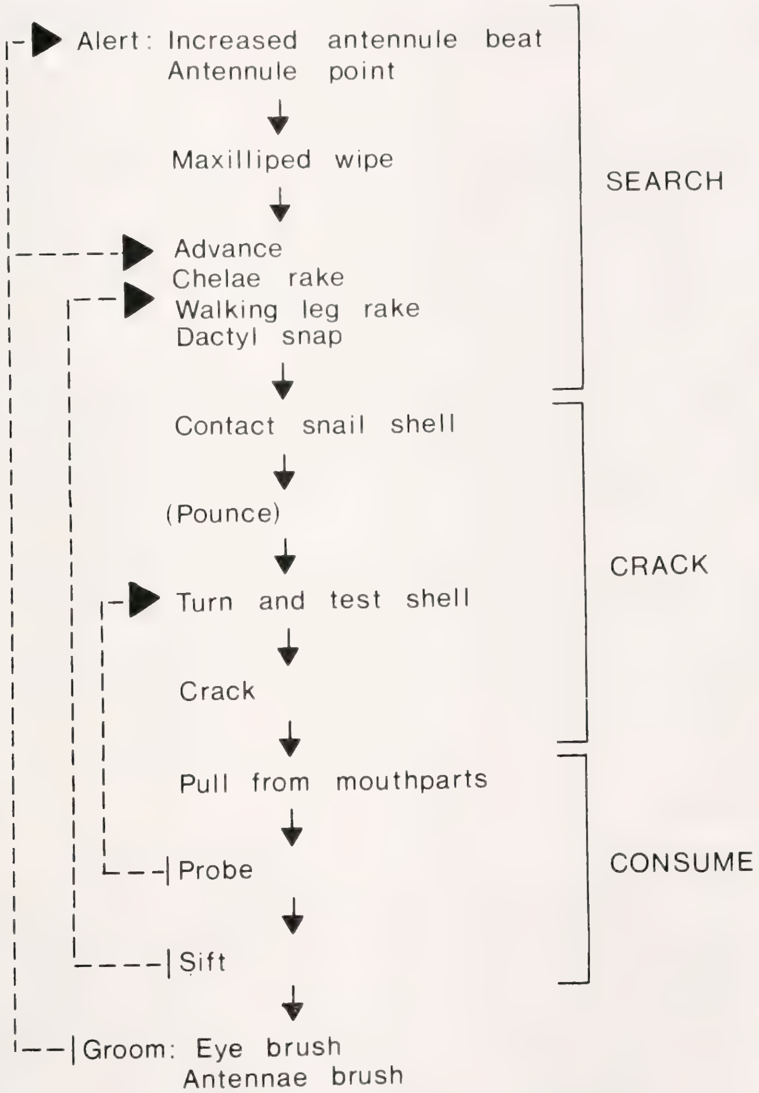

Alert: Increased antennule beat
Antennule point
Maxilliped wipe
Advance
Chelae rake
Walking leg rake
Dactyl snap
Contact snail shell
(Pounce)
Turn and test shell
Crack
Pull from mouthparts
Probe
Sift
Groom: Eye brush
Antennae brush
SEARCH
CRACK
CONSUME

FIGURE 2. Sequence of crab (*Carcinus maenas*) predatory behavior. Dashed lines indicate points at which crabs may return to search or cracking behavior after having begun to consume a snail. This greatly lengthens the consume phase and increases the time available for intact snails to hide.

swept across the substratum with a semicircular swiping motion. While raking, the dactyl of the claw opens and closes (dactyl snap, Fig. 2).

Crack phase. Crabs begin their attack after contacting the snail shell with walking leg or claw. The crab may simply pull the snail from the substratum and bring it to the attack position in front of the mouthparts. Or the crab may suddenly pounce on the snail, pinning it between the carapace and substratum and then pushing the shell forward toward the mouthparts with the walking legs. Once the shell is in front of the mouthparts, the crab turns the shell over with the chelae, pausing to insert the dactyl of the claw into the shell aperture (probe, Fig. 2). The crab then removes the dactyl from the aperture and resumes turning the shell, stopping occasionally with one claw around the shell spire and the other supporting the shell. The third maxillipeds help support the shell during this turning and testing.

If the snail is small relative to crab size, the crab quickly crushes the shell with a claw or breaks off the top of the spire. If the shell is too large to crush, the crab uses alternative methods to expose the snail body; either chipping the outer lip of the aperture until the operculum is no longer flush against the shell and then grasping the snail body behind the operculum with one claw while the other claw tugs the shell in the opposite direction; or gradually chipping away the side of the shell. Either of these techniques requires further cracking of the shell after the first mouthful of snail tissue has been taken.

Consume phase. Once the snail body is exposed, the shell is held up to the mouthparts, supported by both chelae, and the mandibles and maxillipeds tear off bits of flesh. A small cloud of fluid appears around the mouthparts. While mouthparts grip the snail body, the shell is pulled away with the claws, exposing more snail body, until it is consumed. Occasionally the shell is dropped when the snail is only partially consumed but the crab is unable to crack more of the shell.

If the shell has been crushed or broken, the crab picks up the fragments again (sift, Fig. 2) after consuming the snail body. At the end of the "consume phase" the crab sits quietly, resumes searching, or grooms. A summary of the entire predation sequence appears in Figure 2.

In the field, crabs were usually discovered holding periwinkles in the "attack" position, but on one occasion the entire sequence of feeding behavior (search through consume) was observed in a tide pool.

Crab predation: laboratory experiments

Attention was focused on two periods of this predatory behavior to test the utility of snail alarm behavior in preventing crab predation. The two periods were the "search phase" (time between a crab's becoming alerted to the presence of a snail and its attack on the shell); and the "consume phase" (the interval between first injury to snail body and the start of a search for the next snail victim). If responding to snail juice helps snails avoid crab attack, then crabs should require more time to locate and attack sheltered than exposed snails. Also, the response time of snails to snail juice should be less than or equal to the duration of the "consume phase" of crab feeding behavior.

Crabs found and began attacking exposed snails in approximately 4 min, but required longer than 16 min to discover and begin attack on snails in crevices ($P \leqslant 0.005$, Wilcoxon two-sample test, Table I). Only three of six crabs tested

TABLE I

Results of tests comparing crab predation on L. littorea *in crevices or exposed on rock surfaces: Means ± one standard error. Crab size (carapace width in mm) exposed trials: 47.74 ± 1.15; sheltered trials: 47.44 ± 1.46. Snail size (shell length in mm) exposed: 9.83 ± 0.16; sheltered: 9.59 ± 0.19.*

Variable	Snail location		
	Exposed	Sheltered	
Time to crab alert (sec)	31 ± 14	25 ± 8	
range	(1–60)	(1–90)	
Time from crab alert to attack* (sec)	240 ± 79	972 ± 110	$P \leqslant 0.005$
range	(13–465)	(490–1199$^+$)	

* Trial terminated at 20 min even if crab hadn't yet attacked.

with sheltered snails were able to find snails and attack within the 20 min limit of a trial. Crabs became alerted (signaled by antennule pointing) to snail presence equally quickly in both cases, but took longer or were unable to find sheltered snails. Also, once a crab's walking legs or claws had contacted snails in crevices, crabs seemed to have difficulty performing the claw movements required to extract snails from crevices. The time required to consume individuals of *L. littorea* depended on snail size (shell length). The regression equation relating snail size and time required to consume snails was: ln consume time = − 3.28 + 0.488 shell length, $R^2 = 0.40$, $N = 16$. Crabs took longer to consume medium-sized snails than small snails ($P \leqslant 0.05$, analysis of variance). This difference reflects different methods of attack on the two size classes of snail. All crabs consuming medium-sized snails interrupted actual feeding on snail tissue to resume attack on the shell. Small snails' shells were usually crushed immediately. Large snails were attacked (65%) but none consumed (Table II).

DISCUSSION

The size, shape, and structure of gastropod shells is often considered a snail's single or primary defense against shell-destroying predators such as birds, fish,

TABLE II

Crab predation success and the amount of time required to consume small, medium-sized, and large individuals of L. littorea.

	Snail size			
	Small	Medium	Large	
Number of snails presented	17	17	17	
Number (proportion) attacked	13 (0.76)	14 (0.82)	11 (0.65)	
Number (proportion) consumed	8 (0.47)	8 (0.47)	0 (0.00)	
Consumed snails				
Snail shell length (mm)	7.88 ± 0.20	10.53 ± 0.29	—	
Crack phase duration (sec)	30 ± 6	1164 ± 442	—	
range	(2–45)	(25–3625)		
Consume phase duration (sec)	134 ± 52	594 ± 277	—	$P \leqslant 0.05$
range	(15–480)	(163–2505)		

and decapod crustacea (Heller, 1976; Vermeij, 1974, 1976, 1978; Vermeij and Covich, 1978; Hughes and Elner, 1979; Zipser and Vermeij, 1978). In this paper I have assembled evidence for an alarm response of *L. littorea* and its function as a complementary antipredator device. To test the hypothesis that alarm behavior in this snail is an antipredator adaptation, answers to two questions were sought: Do crushing predators prey on *L. littorea* in the field? Is the snail's alarm behavior adapted to predator search and feeding behavior? Answers were derived from laboratory and field observations of crab predation, and from results of field studies of snail alarm behavior. Although further analysis of this behavior would require identification of the alarm substance, such tests were not included in this study.

Both direct and circumstantial evidence suggest that crabs feed on periwinkles in the field. *Carcinus* was observed eating *L. littorea* in tide pools and a stony-bottomed cove. The abundance of broken shells found with shell injuries matching shell damage known to have been inflicted by *Carcinus* in the laboratory suggests that crab predation is not a rare event.

Three characteristics of snail alarm behavior seem adapted to defense against the search and feeding behavior of *Carcinus*: the form of the alarm response, the means by which alarm is communicated, and the time taken by snails to hide.

Individuals of *L. littorea* responded to juices of crushed conspecifics by increasing crawl velocities and moving toward rock crevices and under macroalgae fronds. Thus, the result of alarm behavior is movement to sites where snails are less likely to be stumbled upon by crabs. A snail in a sheltered site is more likely to avoid detection or attack than a snail exposed on a rock surface (Vermeij, 1974) or on the tide pool floor. In the present study it was found that crabs required more time to find periwinkles in crevices and were less successful in attacking once these sheltered periwinkles were found. It is likely that sites under rocks provide a similar refuge.

The majority of gastropod species tested by Snyder (1967), including the mud snail *Ilyanassa obsoleta*, responded to conspecific juice with self-burial. In the laboratory, buried individuals of *I. obsoleta* were attacked by *Carcinus* less frequently than were mud snails exposed on the surface (Ashkenas and Atema, 1978). Responding to a chemical signal is adaptive in defense against activities of a nocturnal tide pool predators such as *Carcinus*.

It is not immediately obvious that a gastropod could avoid being consumed by simply crawling away from its predator or by moving to sheltered sites, since snails are notoriously slow creatures. The key to understanding why this strategy works is knowledge of the predator's feeding behavior and the type of refuge sought by snails. *Carcinus* uses different techniques to attack and devour bivalve and gastropod prey depending on prey size (Elner, 1978; Kitching *et al.*, 1966; Hughes and Elner, 1979; Zipser and Vermeij, 1978). The crab employed a similar size-specific strategy for *L. littorea*. Small periwinkles were crushed and consumed in 3 min, while cracking and eating medium-sized snails took about 26 min longer. Large snails were never successfully consumed in the laboratory. Thus, large individuals of *L. littorea* appear to have a size refuge like that reported for *I. obsoleta* (Ashkenas and Atema, 1978), *L. rudis*, and *L. nigrolineata* (Elner and Raffaelli, 1980).

Individuals of *L. littorea* found sheltered sites in approximately 10 min. Thus, the time required by snails to hide corresponded closely to the amount of time required by crabs to consume medium-sized snails, once first injury to snail tissue had occurred. Although small snails are crushed and eaten too quickly to allow nearby conspecifics time to hide, with increased distance from the predator snails gain time to find shelter.

If the juice of crushed conspecifics signals a real threat to intact snails, crabs must search for a second snail after consuming the first (Snyder, 1967). All 11 crabs which consumed at least one *L. littorea* in the laboratory continued searching behavior after the first snail had been eaten. These crabs had been without food for 7–10 days when tested. In the field *Carcinus* probably feeds more frequently and may never consume more than one snail per feeding period. However, the single green crab observed through an entire episode of feeding in the field resumed searching as soon as the first snail was gone. Additional field observations are needed on this aspect of the snail alarm–crab predation relationship.

Of course, crabs are not the only predator of *Littorina* able to release snail juice. Carnivorous whelks (*Thais*), herring gulls, ducks, fish, and lobsters have also been reported to eat *L. littorea* (Pettitt, 1975). The shelter-seeking behavior of the snails may also be an effective defense against visual predators, such as birds. The alarm response would be equally effective against any predator which injures snail tissue, takes more time to consume a snail than snails require to hide, and consumes more than one snail per feeding period. However, the volume and mixing of water along the shore at high tide is so much greater than the volume and mixing in a tide pool at low tide that stimulus molecules probably do not reach concentrations sufficient to affect any snails but those a few millimeters from the crushed snail. Thus, alarm responses may only occur in tide pools or other areas where water is shallow and still, such as a tidal marsh at low tide.

In an evolutionary race between shell-crushing predators and their gastropod prey, the evolution of elaborate shell ornamentation, short shell spires, narrow opercula, or thick shell walls may be one line of defense for a snail (Vermeij, 1978). However, these morphological adaptations are more often found among tropical than among temperate species. It appears that a complementary first-line strategy for the temperate *L. littorea* is behavioral defense: alarm behavior which helps a snail avoid detection or attack. Perhaps the most interesting challenge remains: the unraveling of interactions among all selective pressures which together determine whether shell structure, alarm behavior, or a combination of the two evolves for snail defense.

The section on snail alarm behavior was first prepared as an undergraduate thesis under the supervision of Beverly Greenspan and James Moulton, Department of Biology, Bowdoin College. William and Barbara Hadlock made possible the frequent field site visits. I appreciate Tom Seeley's interest and assistance with pilot field studies. The comments of Jelle Atema, J. Stanley Cobb, and Tom Seeley on different drafts of the manuscript improved its final form. This work was supported in part by the Lerner Fund for Marine Research, American Museum of Natural History; and the Elliott Fund of Bowdoin College.

SUMMARY

Individuals of *Littorina littorea* in rocky intertidal pools crawled to pool sites where they were less visible (into rock crevices; under rocks and macroalgal fronds) when either crushed conspecifics or juice from crushed conspecifics was added to

these pools. A significant proportion of snails hid in 10 min or less; individual snails in one pool tested quadrupled their crawling velocities after snail juice was added.

Field observations and laboratory experiments tested the hypothesis that this alarm behavior helps *L. littorea* avoid being eaten. Green crabs (*Carcinus maenas*) were observed consuming individuals of *L. littorea* in tide pools at night and along the shore at high tide during the day. In the laboratory, crabs required more time to locate and attack periwinkles in rock crevices than periwinkles on rock surfaces. The amount of time required to consume specimens of *L. littorea* depended on snail size (shell length), reflecting different methods of attack by crabs. Small snails (< 9.0 mm) were crushed, then consumed in approximately 2 min 30 sec. Crabs could not consume large snails (> 18.0 mm), but destroyed medium-sized snails ($\geqslant 9.0, \leqslant 18.0$ mm) by cracking the shell, tearing off bits of tissue, then resuming shell cracking to expose more snail tissue. This required a mean time of 9 min 54 sec once first injury to snail tissue had occurred, which approximately equals the 10-min response time of snails exposed to crushed snail or snail juice in the field. These findings indicate that the alarm response of *L. littorea* serves in defense against *Carcinus maenas*.

LITERATURE CITED

ATEMA, J., AND G. BURD, 1975. A field study of chemotactic responses of the marine mud snail, *Nassarius obsoletus*. *J. Chem. Ecol.*, **1**: 243–251.

ATEMA, J., AND D. STENZLER, 1977. Alarm response of the marine mud snail, *Nassarius obsoletus*: biological characterization and possible evolution. *J. Chem. Ecol.*, **3**: 173–187.

ASHKENAS, L., AND J. ATEMA, 1978. A salt marsh predator–prey relationship: attack behavior of *Carcinus maenas* (L.) and defenses of *Ilyanassa obsoleta* (Say). *Biol. Bull.*, **155**: 426.

ELNER, R. W., 1978. The mechanics of predation by the shore crab, *Carcinus maenas* (L.) on the edible mussel, *Mytilus edulis* L. *Oecologia*, **3**: 333–344.

ELNER, R. W., AND D. G. RAFFAELLI, 1980. Interactions between two marine snails, *Littorina rudis* Maton and *Littorina nigrolineata* Gray, a predator, *Carcinus maenas* (L.), and a parasite, *Microphallus similis* Jägerskiold. *J. Exp. Mar. Biol. Ecol.*, **44**: 151–160.

VON FRISCH, K., 1938. Zur Psychologie des Fisch-Schwarmes. *Naturwissenschaften*, **26**: 601–606.

HELLER, J., 1976. The effects of exposure and predation on the shell of two British winkles. *J. Zool. London*, **179**: 201–213.

HOWE, N. R., AND Y. M. SHEIKH, 1975. Anthopleurine: a sea anemone alarm pheromone. *Science*, **189**: 386–388.

HUGHES, R. N., AND R. W. ELNER, 1979. Tactics of a predator, *Carcinus maenas*, and morphological responses of the prey, *Nucella lapillus*. *J. Anim. Ecol.*, **48**: 65–78.

KEMPENDORFF, W., 1942. Über das Fluchtphänomen und die Chemorezeption von *Helisoma* (*Taphius*) *nigricans* Spix. *Arch. Molluskenk.*, **74**: 1–27.

KITCHING, J. A., L. MUNTZ, AND F. J. EBLING, 1966. The ecology of Lough Ine. XV. The ecological significance of shell and body forms in *Nucella*. *J. Anim. Ecol.*, **35**: 113–126.

KULZER, E., 1954. Untersuchung über die Schreckreaktion bei Erdkrotenquappen (*Bufo bulo* L.). *Z. Vergl. Physiol.*, **36**: 443–463.

MACKIE, A. M., AND P. T. GRANT, 1974. Interspecies and intraspecies chemoreception by marine invertebrates. Pages 105–141 in P. T. Grant and A. M. Mackie, Eds., *Chemoreception in marine organisms*. Academic Press, London.

PETTITT, C., 1975. A review of the predators of *Littorina*, especially those of *L. saxatilis* (Olivi) (Gastropoda: Prosobranchia). *J. Conchol.* **28**: 343–357.

SNYDER, N. F. R., 1967. An alarm response of aquatic gastropods to intraspecific extract. *Cornell University Agricultural Experiment Station Memoir* **403**, 126 pp.

SNYDER, N. F. R., AND H. A. SNYDER, 1970. Alarm response of *Diadema antillarum*. *Science*, **168**: 276–278.

SNYDER, N. F. R., AND H. A. SNYDER, 1971. Defenses of the Florida apple snail, *Pomacea paludosa*. *Behaviour*, **40**: 175–215.

SOKAL, R. R., AND F. J. ROHLF, 1969. *Biometry: the principles and practice of statistics in biological research.* W. H. Freeman, San Francisco, 776 pp.

STENZLER, D., AND J. ATEMA, 1977. Alarm response of the marine mud snail, *Nassarius obsoletus:* specificity and behavioral priority. *J. Chem. Ecol.*, **3**: 159–171.

UNDERWOOD, A. J., 1973. Studies on the zonation of intertidal prosobranchs (Gastropoda: Prosobranchia) in the region of Heybrook Bay, Plymouth. *J. Anim. Ecol.*, **42**: 353–372.

VERMEIJ, G. J., 1974. Marine faunal dominance and molluscan shell form. *Evolution*, **28**: 656–664.

VERMEIJ, G. J., 1976. Interoceanic differences in vulnerability of shelled prey to crab predation. *Nature*, **260**: 135–136.

VERMEIJ, G. J., 1978. *Biogeography and adaptation: patterns of marine life.* Harvard University Press, Cambridge, Mass., 332 pp.

VERMEIJ, G. J., AND A. P. COVICH, 1978. Coevolution of freshwater gastropods and their predators. *Am. Nat.*, **112**: 833–843.

ZIPSER, E., AND G. J. VERMEIJ, 1978. Crushing behavior of tropical and temperate crabs. *J. Exp. Mar. Biol. Ecol.*, **31**: 155–172.

Reference: *Biol. Bull.,* **159**: 280–294. (October, 1980)

THE FORMATION AND EARLY DIFFERENTIATION OF SEA URCHIN GONADS

MARGARET S. HOUK AND RALPH T. HINEGARDNER

Division of Natural Sciences, University of California, Santa Cruz, California 95064

Despite extensive use of sea urchin gametes in developmental biology, few modern studies deal with the origins and differentiation of the gametes from the primary germ cells (PGCs). All published studies are from the last century or the early part of this one, the major ones being those by Hamann (1887); Prouho (1887); Cuenot (1891); Russo (1894); and MacBride (1903). This and other work on gonad development has been summarized by Delavault (1966). Most authors agree that the gonadal or genital primordium, variously called the genital rudiment, genital cord, or sexual bud, is identifiable soon after metamorphosis from pluteus larva to juvenile urchin as an accumulation of large cells located in the dorsal mesentary. This mesentary, which forms from the apposition of the walls of the left and right posterior coeloms of the larva (the somatocoels of the adult), adheres to the aboral body wall in the vicinity of the madreporite and extends into the main body cavity to form the boundary between the left and right somatocoels. The dorsal mesentary also supports the stone canal, axial organ, and parts of the gut. Published studies indicate that the genital primordium later spreads circumferentially around the inside aboral surface and eventually forms a circular cord of cells, the genital rachis, which competely encircles the periproct. The gonads differentiate from five swellings on the rachis, with one gonad forming in each interradius.

We initiated this study to determine the accuracy of the earlier observations and to expand upon them through electron microscopy. We have confirmed the location of the gonadal primordium in the dorsal mesentery and its subsequent development into genital rachis and gonads. We differ, however, from most previous investigators in our interpretation of some features of the process. Our ultrastructural examination of the gonadal primordium sheds additional light not only on the origin of the gametes, but on the origin and differentation of the gonadal accessory cells (sometimes called the nutritive phagocytes).

Materials and Methods

Selection of material

The sea urchins we used were out-crossed laboratory-raised individuals of the species *Lytechinus pictus,* grown according to the methods of Hinegardner(1969). Juvenile urchins selected for tissue sectioning ranged from 0.3 to 6.0 mm in outer diameter and in age from 3 days post metamorphosis to 8 months post metamorphosis. We have been able to identify the genital cells in all stages up to the time when recognizable eggs and sperm are found.

Preparation for light microscopy

Juvenile urchins were fixed in ethanol-acetic acid, 3:1, and dehydrated in an ethanol series. Urchins under 3 mm in diameter were embedded for serial sectioning first in celloidin and then in paraffin according to the method of Akesson (1961). Larger juveniles were embedded directly in paraffin. Five μm serial sections were used. After removal of the paraffin with xylene, sections were passed from 100 to 90% ethanol and stained for 20 sec in Unna-Pappenheim methyl-green pyronin stain (Gurr, 1965). They were then washed in water for 1 min, blotted to remove excess water, and passed directly to 100% ethanol for 10 sec of agitation. Rapid passage through water and ethanol was necessary to prevent loss of stain. Cell counts at various growth stages were made from *camera lucida* drawings using semi-transparent paper so that tracings could be overlaid for comparison. For each individual in which germ cells were counted, all sections containing any germ material were traced and compared with adjacent sections to avoid counting the same cell more than once.

Preparation for transmission electron microscopy

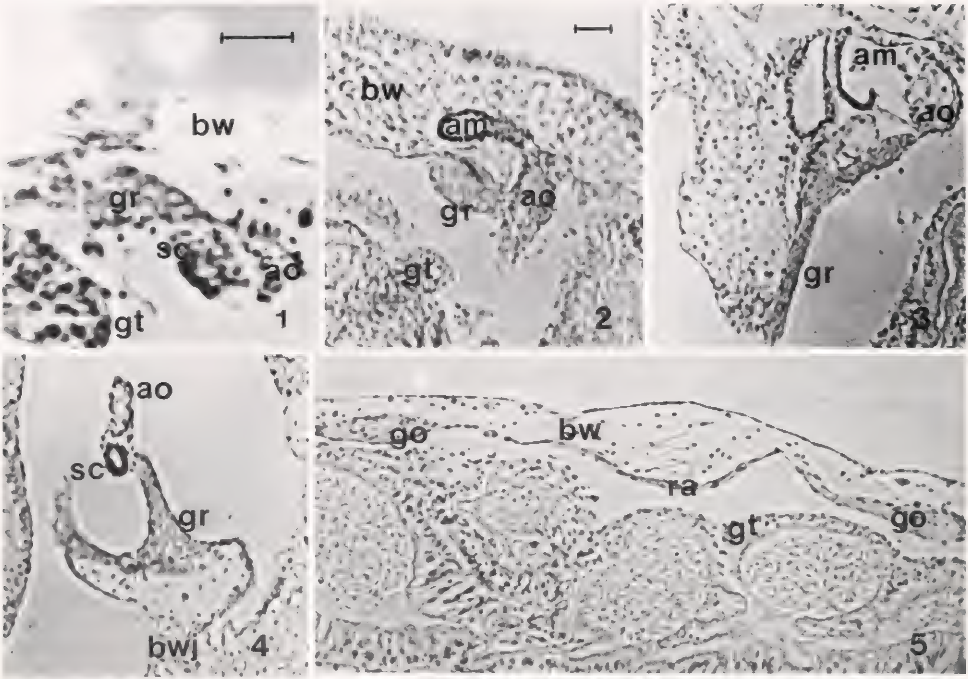
Urchins were prepared for fixation by several methods depending on their size. Gonads from mature individuals ($>$ 10 mm in diameter) were excised from the body cavity prior to fixation. Juvenile urchins were bisected through their largest diameter. The aboral half, containing the germ cells, was then inverted to form a "bowl" and flushed several times with fixative prior to immersion in fresh fixative. The desired tissues were then dissected from the urchin. Juveniles under 1.5 mm were fixed whole.

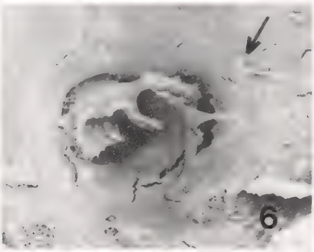
Most material was fixed in 1.5% glutaraldehyde in sea water for 1 hr. All manipulations were carried out at pH 7.0–7.6 and at 4° C. A few samples were fixed according to the method of Bal *et al.* (1968). Tissues were next washed in several changes of sea water and post-fixed for 2–2.5 hr in 1% osmium tetroxide in sea water. Dehydration was carried out in an acetone series. Tissues were embedded in Spurr's resin (Spurr, 1969). Material from early juvenile stages had to be decalcified because the minute germinal primordia could not be removed by dissection. Decalcification was carried out on material fixed overnight in 1.5% glutaraldehyde followed by osmium tetroxide as described above. Samples were either decalcified in ascorbic acid according to the method of Dietrich and Fontaine (1975) or in 5% EGTA in 0.1 M sodium phosphate buffer at pH 7 for 1 hr.

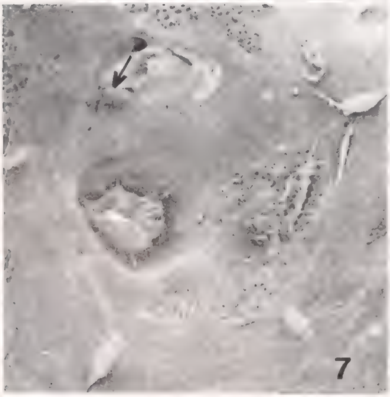
Blocks of tissue were first sectioned (0.5 μm) for purposes of orientation. These thick sections were stained for light microscopy with 1% toluidine blue in 1% sodium borate. Thin sections were cut with a Porter-Blum MT-2 ultramicrotome using a glass knife. Sections were stained for 30 min with Millipore filtered 2% uranyl acetate and 5 min with Reynolds' lead citrate (Reynolds, 1963). The electron microscope was a JEOL GEM 100B.

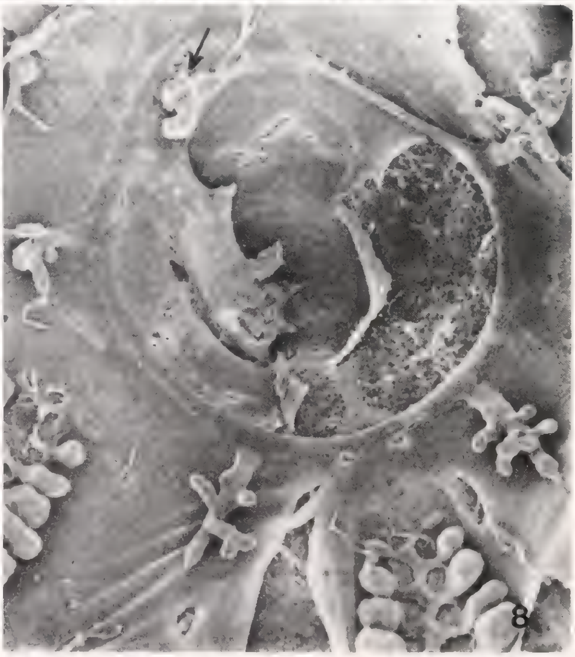
Preparation for scanning electron microscopy

Juvenile urchins ranging from 3 to 7 mm in diameter were prepared for scanning electron microscopy by bisecting the specimen equatorially slightly on the aboral side of the greatest diameter. The aboral part was flooded with 1.5% glutaraldehyde in sea water and any remaining pieces of gut carefully dissected out under a dissecting microscope. Specimens were dehydrated in an ethanol series, substituted with amyl acetate, then critical-point dried in carbon dioxide (Anderson, 1951), coated with carbon and gold and examined with a JEOL JSM-2 scanning electron microscope.


bw
gr
sc
ao
gt
1
bw
am
gr
ao
gt
2
am
ao
gr
3
ao
sc
gr
bwj
4
go
bw
ra
gt
go
5







FIGURES 1–5. Paraffin sections through the germinal tissues of juvenile sea urchins. Figure 1—3-day-old juvenile 0.3 mm in diameter. Figure 2—Month-old juvenile 1.0 mm in diameter. Figure 3—Three-month-old juvenile, 2.0 mm in diameter, sectioned at a 45° angle to the oral-aboral axis. Figure 4—Three-month-old juvenile, 2.0 mm in diameter, sectioned circumferentially. The genital primordium *gr* is suspended between the aboral body wall *bw* and stone canal *sc* and closely associated with the axial organ *ao* near where the gut *gt*

TABLE I

Number of primordial germ cells present in the juvenile sea urchin at different post-metamorphosis ages and test diameters

Days past metamorphosis	Outer diameter of test (mm)	No. urchins counted	Average no. germ cells ± SD
1–3	0.3	5	20.8 ± 5.4
29	0.68	1	21
29	0.85	3	36.0 ± 5.3
29	1.0	3	29.33 ± 3.8
29	1.2	3	60.3 ± 12.0
93	1.5	1	60
93	2.0	2	101.0 ± 26.9
Unknown	2.7	2	1000 approx.

RESULTS

Differentiation prior to gonad formation

In the newly metamorphosed sea urchin, the primordial germ cells (PGCs) of the genital primordium are identifiable in serially sectioned material as a group of pyrinophilic cells with large (5 μm) nuclei containing one or two nucleoli. The PGCs remain suspended in the mesentery between the stone canal and the inner surface of the aboral wall throughout early juvenile development (Fig. 1). By the time the urchin reaches a diameter of 1 mm (approximately 1 month after metamorphosis), the germ cells have clustered into a distinct group of cells within the germinal primordium (Fig. 2), and are surrounded by a layer of squamous epithelium originating from the mesentery. No coelomic cavity has been identified in association with this group of cells.

Two types of cells, as determined by nuclear morphology, can be found in the genital primordium. The first are the primary germ cells found in the mesentery at the time of metamorphosis. The other type, which we call the presumptive accessory cells, has a nucleus of about 2.5 μm in diameter that stains more heavily than the first type with methyl-green. These cells were not seen in the dorsal mesentery of newly metamorphosed urchins, and their origin is not known.

We found the size of juvenile urchins, rather than their post-metamorphic age, to be the more reliable indicator of the degree of differentiation of the genital tissues (Table I), and therefore include test diameter as an indication of the developmental

---

meets the anus. *am* = ampulla of the left anterior coelom. *bwj* = junction of projecting part of body wall with the main body wall. Figure 5 shows the aboral region of a juvenile 2.5 mm in diameter. Two of the gonadal primordia *go* are connected by part of the genital rachis *ra*. Bars = 10 μm. Figures 2–5 are at the same magnification.

FIGURES 6–8. Scanning electron micrographs of the inner perianal region of juvenile sea urchins. Most of the gut has been removed. Figure 6 shows an individual 1.5 mm in diameter. The torn edges of the intestine surround the anal area at the left corner, while the axial organ and attached dorsal mesentery containing the genital primordium appear at upper right (arrow). Figure 7 depicts an urchin 3.5 mm in diameter with unbranched gonads, four of which are arranged in a circle, one each right and left, and two at the bottom of the figure. The axial organ appears just above the gut in the upper left corner (arrow). Figure 8 shows the gonads of an urchin 5.0 mm in diameter. Four gonads and axial organ (arrow) are shown, oriented as in Figure 7. All three figures are at the same approximate magnification (× 55–60). Field width for 6 is 1.04 mm, for Figure 7 is 1.65 mm, and for Figure 8, 2.02 mm.

stage of our material. In urchins as small as 1.5 mm in diameter, the genital primordium has already begun to broaden and elongate within the dorsal mesentery due to an increase in the number of PGCs (Table I). At this stage genital cells occupy most of the dorsal region of that mesentery. Subsequent gonad development is easiest to understand by visualizing the primordium as something like a letter I lying in the mesentery. As the urchin grows the PGCs migrate to the top of the I, where the dorsal mesentery joins the dorsal body wall. The PGCs then begin to migrate laterally, converting the I to a T-shaped structure. The arms of the T gradually elongate and its stem shortens. Evenutally the stem disappears and the elongating arms form a ring around the periproct. This ring is called the genital rachis (Hamann, 1887). Its diameter is about 1/4 the diameter of the urchin.

Figures 3 and 4 illustrate sections from urchins 2.0 mm in diameter, which have just begun to form a rachis. The section in Figure 3 is approximately perpendicular to the oral-aboral axis and shows the location of the band of genital tissue relative to the left anterior coelom (axocoel), the axial organ, and the stone canal. The rachis lies along part of the body wall which projects into the coelomic space due to the presence of the axocoel and its associated structures. Figure 4 is oriented approximately 45° to Figure 3 and shows the arms of the T-shaped genital primordium. There is a continuum of cells throughout the primordium, with no separation between those beginning to form the rachis and those still within the mesentery. The genital primordium in Figure 4 appears to be connected to the main body wall by only a narrow piece of tissue, but this appearance is due to the angle of the section.

After the rachis has formed, five thickenings appear in it, one in each interradius. These eventually contain most of the PGCs and ultimately give rise to the five gonads. Two such forming gonads and a large portion of the genital rachis, from an urchin 2.5 mm in diameter, are shown in Figure 5. Both the rachis and the developing gonads are surrounded by a layer of epithelium which seems to be derived from the epithelium for the mesentery in which the genital primordium was first found, and thus to be coelomic in origin. Both cell types found in the genital primordium are present in the rachis and in the developing gonads. In larger urchins, with differentiated gonads, the solid cord of PGCs in the rachis has disappeared.

Differentiation of the gonads

No gonads are visible in urchins 1.5 mm in diameter (Fig. 6). Unbranched gonads are present in a 3.5-mm-diameter urchin (Fig. 7). A raised circular area on the aboral wall connects the bases of the gonads, indicating the presence of the genital rachis. Figure 8 shows this area of an animal 5.0 mm in diameter. The gonads have branched several times. That a rachis still exists, even in an individual in which all the germ cells have undoubtedly migrated into the gonads, can be seen in the upper right portion of Figure 8, where the rachis has torn free from the underlying epithelium. We can therefore corroborate the persistence of the now empty rachis as the aboral ring canal, as reported by Cuenot (1891) and Russo (1894). Further branching continues to occur as the gonads grow, until the aboral part of the coelom contains a mass of branching tubules. Each gonad is surrounded by an epithelial layer continuous with the lining of the perivisceral coelom. Besides separating the gonad from the main body cavity, this cell layer forms mesenteries which attach it to the aboral body wall. No gonoduct is present at this time.

Sexual differentiation of the gonad is first apparent with the presence of recognizable oocytes and spermatids. These appear at about the same time or shortly after the gonads begin to branch. By the time male urchins reach a diameter of 5 mm their gonads contain mature spermatozoa in the central cavity, but they usually cannot be induced to spawn. Females 5–6 mm in diameter have previtellogenic oocytes up to 25 μm in diameter (mature eggs are about 110 μm in diameter). The structure of the female gonad at this stage does not differ substantially from that of published descriptions of the mature gonad of other species (Holland and Giese, 1965; Chatlynne, 1969; Davis, 1971; and Pearse, 1969a, b). A gonoduct leads from the central cavity of the gonad to the outer wall of the test. It consists of a single layer of cuboidal cells of unknown origin.

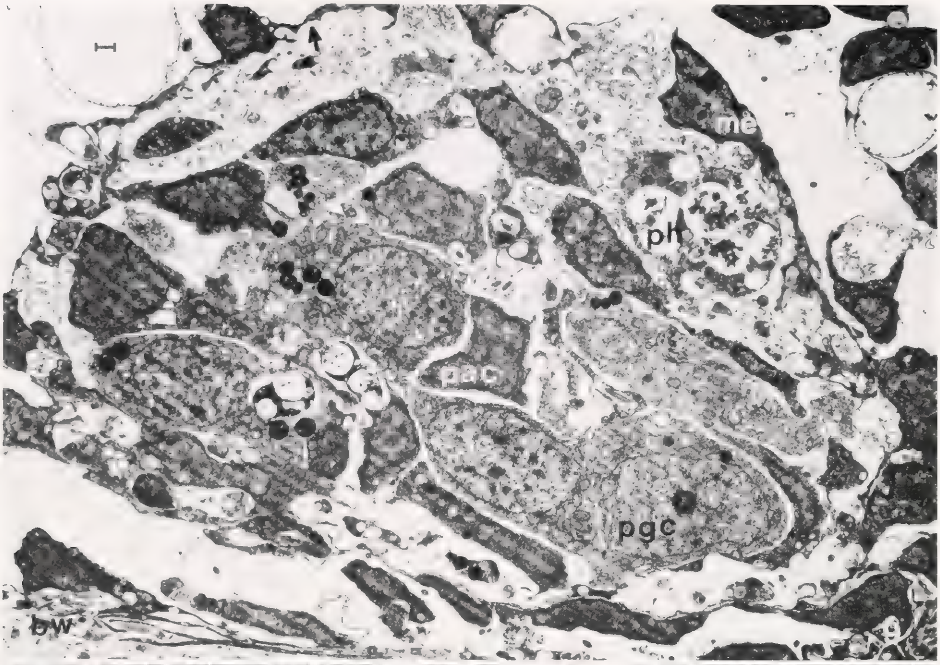
Ultrastructure of the genital tissues

Three stages in the development of the juvenile urchins were selected for ultrastructural studies. These stages were: 1) the genital primordium of newly metamorphosed urchins (0.5 mm in diameter), 2) the older genital primordium, in which the PGCs are rapidly proliferating (1.5 mm), and 3) a sexually undifferentiated gonad (test diameter 3.5 mm). Gonads from sexually differentiated urchins were also studied for comparative purposes and to check our results against published studies where different species and fixation procedures were used.

1. Genital primordium of newly metamorphosed urchin. A tangential section through the genital primordium of a sea urchin with test 0.5 mm in diameter (3 weeks after metamorphosis) is shown in Figure 9. The genital primordium is roughly 33 μm across and is bounded on the outside by an epithelium of ciliated squamous cells. A thick connective-tissue layer lies beneath the epithelium and contains scattered phagocytic cells. The connective-tissue layer is not strictly separated from the underlying cells, and fibrous material similar to that in the connective tissue is often found between these cells. The genital primordium is separated from the body wall by the epithelium of the mesentery and by the peritoneum of the somatocoel. A pronounced basement lamina is found underlying the epithelial cells. The mesentery containing the genital primordium is attached to the body wall *via* the epithelial layers at the right of the figure.

Two cell types are found within the genital primordium. The first are elongate, smooth surfaced, rounded in cross section, and have a round to oval nucleus containing a relatively electron-transparent nuclear matrix. The nucleus is 5–6 μm in diameter. These cells correspond to the primordial germ cells seen with the light microscope. A fibrillar material, presumably chromatin, is fairly evenly distributed within the nucleus. Dense granules about 60 nm in diameter are interspersed among the chromatin material (Fig. 10). The cytoplasm contains the usual cellular organelles, most of which are concentrated in the tapered ends of the cells. Membrane-bound vesicles as large as 0.9 μm in diameter and containing an electron-dense amorphous material are also present (Fig. 9). These are not seen in the larval material we have studied nor in later stages.

Associated with the mitochondria, and sometimes apparently touching their outer membranes, are 100 nm granules containing a dense fibrillo-granular material. These are probably identical to the granules found by Longo and Anderson (1969) in sea urchin spermatogonia. The granules are sometimes clustered (Fig. 11).



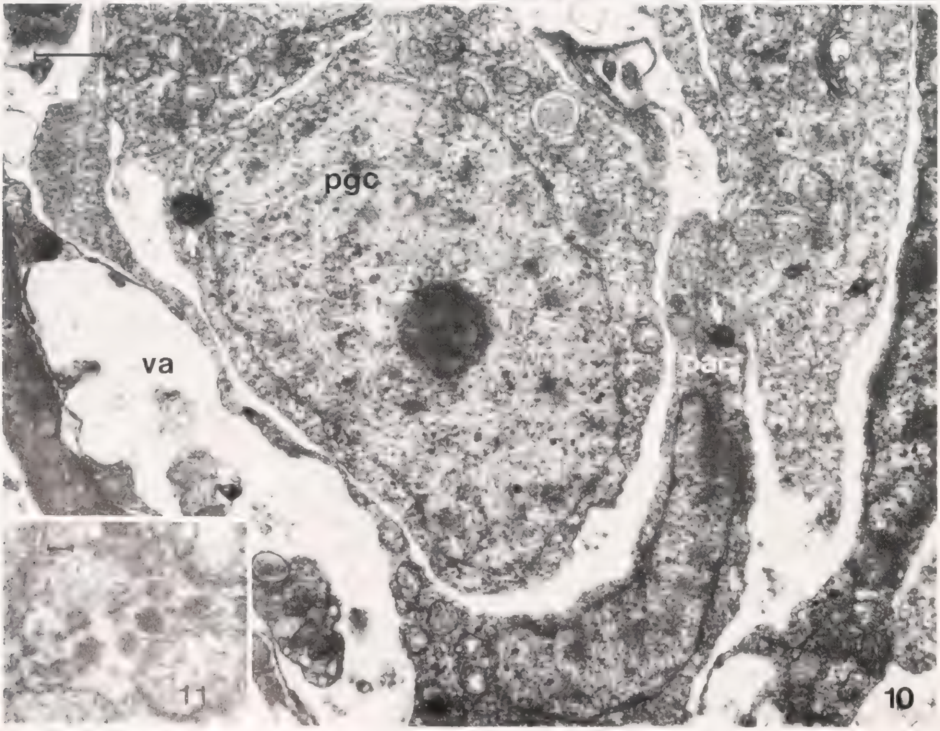


FIGURE 9. Section through the genital primordium of an urchin 0.5 mm in diameter showing the mesentary epithelium *me*, with its underlying basement lamella (arrow), primordial germ cells *pgc*, presumptive accessory cells *pac*, and a phagocytic cell *ph*. The epithelium of the mesentery joins that of the body wall *bw* at the lower right hand side of the figure. Bar = 1 μm.

They are common in these cells as well as those in later stages. Since they seem to be specific to the gamete-forming cells, we will refer to them as *goniosomes.* Amorphous electron-dense material similar to that found in germ cells of other organisms (Eddy, 1975) is also present in the cytoplasm of PGCs.

The cells that we are calling primary germ cells share a number of characteristics with gonial cells, of which they are certainly the precursors. These include: nuclear morphology; the general appearance and contents of the cytoplasm, including goniosomes; and staining characteristics using methyl-green pyronin and toluidine blue.

The second cell type is almost certainly the precursor of the accessory cells of the gonad, which it resembles in both shape and nuclear morphology. These accessory-precursor cells are smaller than the PGCs and are generally angular in cross section, with many elongate cellular processes (Figs. 9, 10). The nucleus is also elongate and irregular in outline. Its matrix is more electron-dense than is the nucleus of the PGC, and areas of condensed chromatin are obvious. Small dark 60 nm granules are present here as they were in the PGCs, and cytoplasmic inclusions are similar. Structures which look like the 100 nm goniosomes common in the PGC have not been found in these cells. The accessory cell primordium also contains a large electron-transparent vacuole (Fig. 10) similar to that found in the accessory cell of the mature gonad (Holland and Giese, 1965). Flagellar cross-sections among the cells of the genital primordium indicate that PGCs and/or accessory-cell precursors are flagellated.

2. *Genital primordium with rapidly proliferating PGCs.* The genital primordium cells of a 1.5 mm urchin are similar to those of the younger animal (Fig. 12). The PGCs have the same general appearance except for the absence of the large membrane-bound vesicles filled with electron-dense material. Mitotic cells can now be found. Otherwise, the PGCs are unchanged.

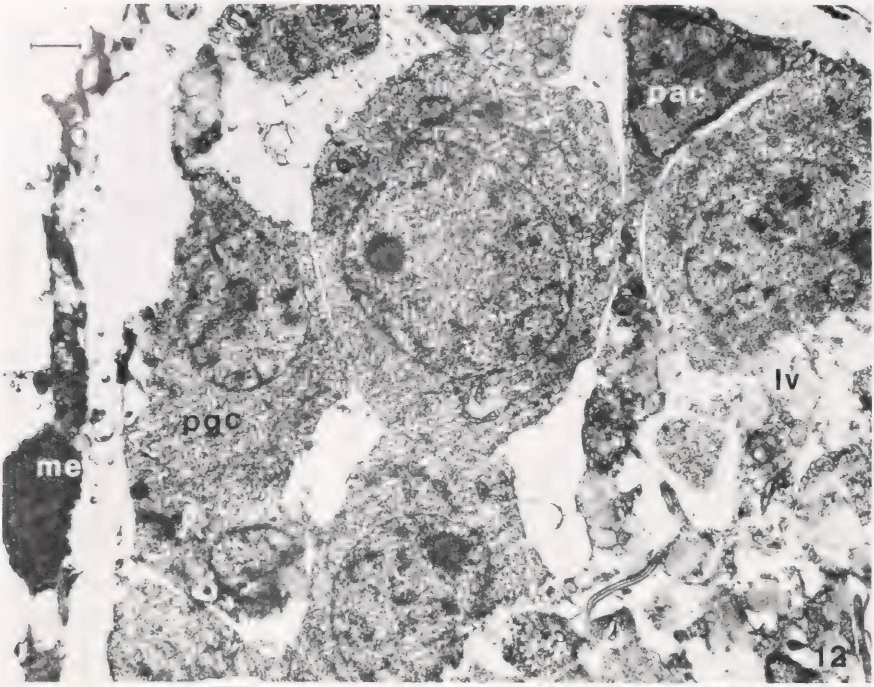
The accessory-cell primordia have now become obvious accessory-cell precursors. The angular nucleus is surrounded by a very thin layer of cytoplasm (Fig. 12). Extensive processes containing large numbers of membrane-bound lipid-like vesicles, similar to those found in the accessory cells of mature gonads, can be seen extending between the PGCs. Glycogen granules, which are abundant in the accessory cells of the mature gonads, are often present in the cellular extensions. The vacuole has elongated. Nuclear morphology remains relatively unchanged and is similar to that of accessory cells of the mature gonad. Flagellar cross-sections are present among these cells.

3. *Sexually undifferentiated gonads.* A cross section of an unbranched gonad from a 3.5-mm-diameter urchin is shown in Figure 13. A many-layered gonad wall surrounds the PGCs. From outside to inside these layers consist of flagellated coelomic epithelium, a connective-tissue layer, a fluid-filled space, a musculo-epithelial layer (also flagellated), and the germinal epithelium. This order is identical to that of the layers in the wall of the mature gonad (Kawaguti, 1965; Davis, 1971). The outer epithelium is continuous with the lining of the perivisceral coelom.

The PGCs dominate the outer germinal layer, and are arranged two or three cells thick. They are closely packed, adhering by means of junctional complexes

FIGURE 10. A primordial germ cell *pgc* in an urchin 0.5 mm in diameter is partially surrounded by an accessory cell *pac*. Both cells contain vesicles with electron dense material (arrows). The pac also has a large vacuole *va*. Bar = 1 μm.

FIGURE 11. Goniosome cluster from a PGC at the same stage shown in Figures 9 and 10. Bar = 0.1 μm.



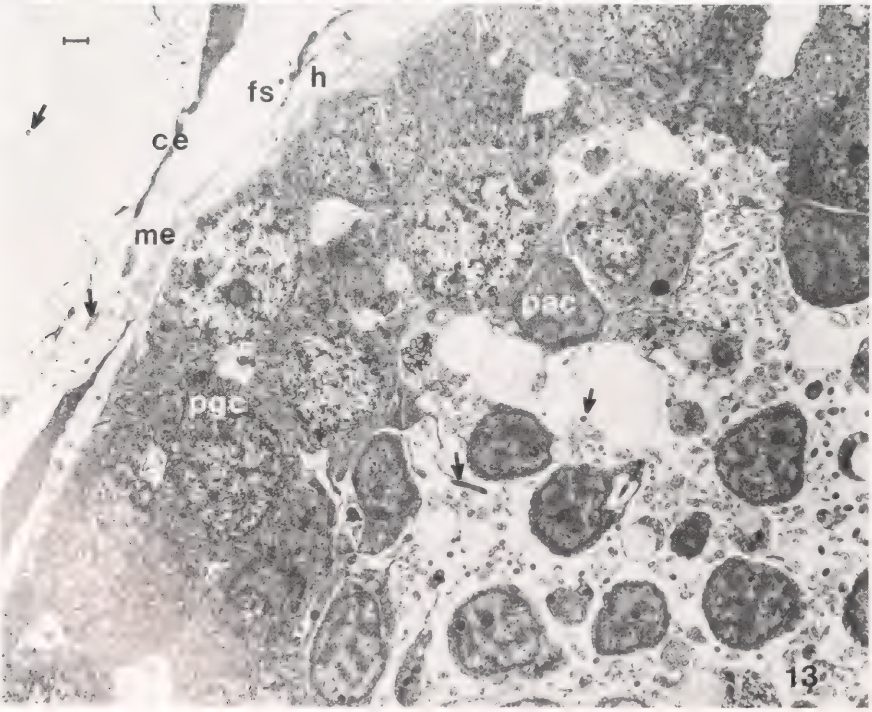


FIGURE 12. Cross section through the genital primordium of an urchin 1.5 mm in diameter, showing primordial germ cells *pgc* and presumptive accessory cells *pac* and the epithelium of the dorsal mesentary *me*. The PACs have long cellular processes containing numerous vesicles filled with lipid-like material *lv*. Flagellar longitudinal sections can be seen in the center portion of the lumen. Bar = 1 μm.

(Fig. 14) and frequently have long cell processes. The nuclei are round and exhibit various degrees of chromosomal condensation, indicative of ongoing mitotic activity. Up to three nucleoli have been seen in a PGC nucleus at this stage. The nucleoli are eccentric and have components with two different degrees of opacity to electrons. The dense cortical part surrounds a less dense fibrillar component. Nuclear pores with annuli are present.

Ribosomes are numerous in the cytoplasm, but endoplasmic reticulum is sparse. Mitochondria, Golgi, and a pair of centrioles are located mainly at the poles of the oval cell. The goniosomes that were described for the genital primordium PGCs are much more numerous at this stage and are scattered among the mitochondria and sometimes initimately associated with them (Fig. 15). Other goniosomes are grouped together in regular arrays (Fig. 14).

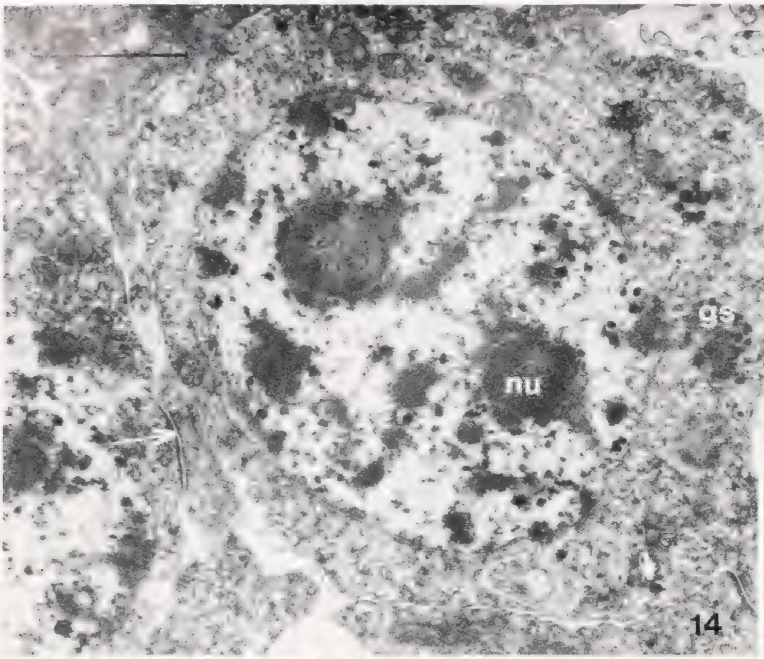
A loosely arranged layer of accessory cells underlies the germinal cells and extends into the central cavity of the gonad. Numerous cell processes, and occassionally, phagocytic cells similar to the one illustrated in Figure 9, are interspersed with these cells. The accessory cells are highly variable in shape and have many cell processes and a large vacuole (Fig. 16). The nuclei look like those of the accessory-cell precursors. Along with the usual organelles, the cytoplasm contains lysosomal-like vesicles identical to those so abundant in the accessory cells of the mature gonad. Glycogen granules and lipid vesicles are more abundant than in earlier stages. Large parts of the cytoplasm appear empty, which is also true of accessory cells of the mature gonad (Verhey and Moyer, 1967). The accessory cells at this stage are smaller than those of the mature gonad and are attached to each other and to the adjacent PGCs via tight junctions (Fig. 16). Sometimes accessory cells can be found completely encircling a PGC. Striated rootlets and flagellar bases have been seen in both the PGCs and the accessory cells, and flagellar cross sections are common in the gonad cavity and between cells (Fig. 13).

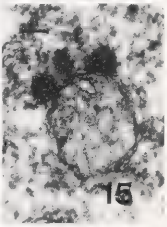
4. *Sexual differentiation of the gonads.* We examined the gonads of urchins 3.3–6.0 mm in diameter to establish a developmental continuum between the younger stages concentrated upon in this study and the ultrastructure of mature gonads as described in published accounts. The earliest indication of sexual differentiation was seen in a female urchin 3 mm in diameter with a gonad that had just begun to form branches. The wall of the gonads differed from sexually undifferentiated stages only in a more extensive development of the muscle fibers of the inner epithelium. Most of the PGCs adhere tightly to each other and are the same size as those of the undifferentiated gonad. However, there are a number of larger cells (10–20 μm in diameter) which have lost their close contacts with their neighbors and have become rounded. One such cell is pictured in Figure 17. The circular nucleus is 7 μm in diameter and centrally located with a single nucleolus. The cytoplasm has inclusions similar to those of the PGC but more evenly distributed. Goniosomes are present, often associated with mitochondria.

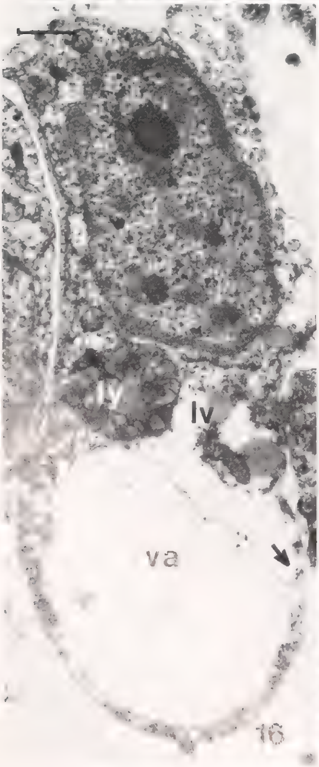
Older individuals (5.0 mm in diameter) show definite sexual differentiation. The solid layer of germ cells apparent in sexually undifferentiated gonads is no longer present, and clusters of gonia are separated from each other by accessory cell processes. In young ovaries a large part of the germinal layer consists of a

---

FIGURE 13. Cross section through the unbranched, sexually undifferentiated urchin 3.5 mm in diameter showing the outer coelomic epithelium *ce* and the muscular epithelium *me* of the gonad wall. The epithelium layers are separated by a fluid filled space *fs*, and a haemal lacuna *h* underlies the muscular epithelium. PGCs form a germinal epithelium next to the haemocoel, while PACs are in and near the central lumen. Ciliary cross sections of outer epithelium, muscular epithelium, and accessory cells are indicated by arrows. Bar = 1 μm.







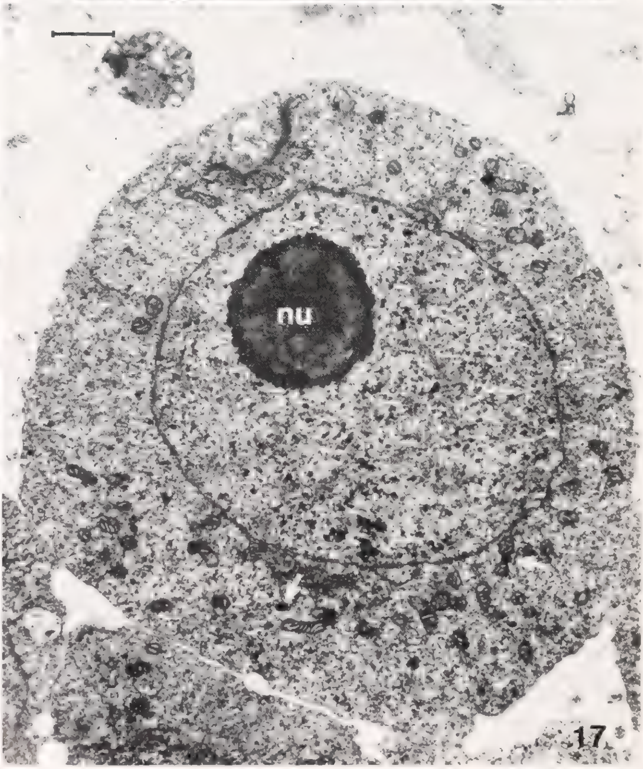


FIGURE 14. A primordial germ cell of the unbranched gonad. Two nucleoli *nu* are present. A cluster of goniosomes *gs* and unclustered goniosomes (short arrows) are in cytoplasm. The PGC adheres to its neighbors by junctional complexes (long arrows). Bar = 1 μm.

single layer of previtellogenic oocytes. Oogonial clusters are widely separated and extend from the gonad wall well into the lumen. Oogonia resemble PGCs in their nuclear morphology and cytoplasmic inclusions, but have a pronounced polarity, with the mitochondria and goniosomes clustered at one end of the oval cell. Polarity seems to be oriented randomly with respect to the ovary wall. Oocytes similar in size and shape to the one pictured in Figure 7 are occasionally seen near the lumen of the ovary. Most of the oocytes, however, are larger, about 25 μm in diameter, and are located in the single layer next to the ovarian wall. The accessory cells resemble those of the mature gonad. Oogenesis has been described in a number of species of sea urchins (*e.g.*, Verhey and Moyer, 1967; Bal *et al.*, 1969; Millonig *et al.*, 1968; Chatlynne, 1972).

Testes in these young urchins are organized similarly to those of mature males. All individuals which could be identified as males already had mature sperm present in their testes lumina. Presumably the spermatogenic cycle is so rapid (less than 12 days in gonads of some species of urchins; Holland and Giese, 1965) that testes containing spermatids and no mature sperm would be hard to find. Spermatogonia are clustered against the wall of the testes and resemble oogonia and PGCs. Spermatogenesis is well described in Longo and Anderson (1969).

Discussion

While most authors agree that the gonads of echinoderms differentiate from a group of cells located in the dorsal mesentery of the newly metamorphosed juvenile, the origin of the cells is disputed. Cuenot (1891) and MacBride (1903) believed that the genital primordia of asteroids, ophiuroids, and echinoids have common origins with the axial organs. In fact, MacBride refers to the axial organ as the genital stolon. Prouho (1887) and Russo (1894) described the genital primordium of sea urchins as arising from the wall of the left anterior coelom independently of the axial organ. MacBride (1903) also proposed an epithelial origin for the germinal cells of sea urchins, since he believed that the genital stolon, from which both the genital primordium and the axial organ supposedly differentiated, arose from the wall of the left posterior coelom of the newly metamorphosed urchin. More recent work by Delavault (1966) on the sea star *Asterina gibbosa* indicates that at least in sea stars, the location of the germ cells in the dorsal mesentery may be secondary and that the genital primordium may descend from mesenchyme cells which migrate to the mesentery during late larval development. Our study of newly metamorphosed juveniles of *Lytechinus pictus* shows that the germ cells are already present in the dorsal mesentery at the time of metamorphosis as a cluster of cells distinct from the axial organ and not connected to the wall of the coelom. Their ultrastructure resembles that of the mesenchymal cells. If these cells do originate from the coelomic epithelium, it must happen during larval development, contrary to MacBride's description for *Echinus esculentus* (1903), or Cuenot's for *Paracentrotus lividus* (1891). While their location and ultrastructural appearance sug-

---

FIGURE 15. Goniosomes closely associated with a mitochondrion in a PGC of the sexually undifferentiated urchin. Bar = 0.1 μm.

FIGURE 16. Presumptive accessory cell in unbranched gonad with lipid vesicles *lv*, lysosomal-like vesicles *ly*, large vacuole *va*, and glycogen granules (arrows). Bar = 0.1 μm.

FIGURE 17. Large oocyte in branched gonad of urchin 3.3 mm in diameter. The single nucleolus *nu* is differentiated into two regions of different opacity to electrons. Goniosomes are present in the cytoplasm (arrows). Bar = 1 μm.

gest a mesenchymal origin for the germ cells, careful study of larval development will be necessary to decide this question.

In other classes of echinoderms, particularly the asteroids, the genital rachis is described as developing from a vesicle which buds off the peritoneum of the left somatocoel and surrounds the germ cells. The space within the vesicle is derived from the coelom. In asteroids this space persists in the wall of the gonad and remains connected with the coelom (Delage and Hérouard, 1903). Such a vesicle does not appear in *Lytechinus pictus*. Rather, the germ cells begin to divide and to travel within the dorsal mesentary to the aboral body wall, extending out into a ring continuous with the mass of cells in the dorsal mesentary. This continuity indicates that the germ cells in the rachis must be between the peritoneum of the coelom and the body wall. The outer covering of both the primordium in the mesentery and the gonad wall is the peritoneal epithelium. The inner musculo-epithelial layer of the gonad wall is not present in the genital primordium stage. The origin of this secondary epithelial layer is not explained by our investigation. The layer appears to be present in the rachis, but ultrastructural examination would be necessary to determine this with certainty. The space between the two epithelia is probably a schizocoel, as suggested by Hamann (1887).

The accessory cells, also called nutritive phagocytes, have been studied in the mature gonad by several authors (Holland and Giese, 1965; Verhey and Moyer, 1967; Masuda and Dan, 1977). These cells are present in both sexes of the adult and change in size and internal constitution during the gametogenic cycle, giving rise to the hypothesis that they are involved in recycling nutrients from phagocytized gametes (Holland and Giese, 1965; Pearse, 1969a, b). Various origins for these cells have been suggested. Some authors have claimed that they originate from the same cells in the gonads as the gonia and therefore fit the classical definition of nurse cells (Miller and Smith, 1931). In our study we have seen that accessory cell primordia are present in the genital primordium soon after metamorphosis and migrate with the PGCs from the genital primordium through the rachis into the gonad. Differentiation into recognizable accessory cells consists primarily of the accumulation of cytoplasmic inclusions such as glycogen granules, lipid-like vesicles, and lysozomal-like vesicles. It begins while the cells are still in the genital primordium. This gradual accumulation of the cytoplasmic structures characteristic of accessory cells leaves little doubt that these cells differentiate from the small-nucleus cells of the genital primordium.

In young juveniles 1 mm in diameter, the cytoplasm of both the PGCs and the presumptive accessory cells contains membrane-bound vesicles filled with an electron-dense material which resembles yolk. These vesicles are seldom seen in either cell type at later stages. Possibly they are diluted as the cells divide. The presence of yolk in the PGCs of sea urchins is a reasonable suggestion, since PGCs of anuran amphibians are known to contain yolk platelets (Blackler, 1958). If these vesicles prove to be generally present in PGCs of larvae as well, they may be useful as cytoplasmic markers for tracing the germ cells back through larval development. While the PGCs and the presumptive accessory cells are distinct from each other in all the stages covered by this study, the presence of yolk-like structures in both cell types suggests they may have a common embryological origin.

While the PGCs share some features with the accessory cells, they are much closer in appearance to gonia. They have similar nuclear morphology, general cell shape, and cytoplasmic inclusions, especially the 100-nm goniosomes which were found at all stages examined and only in germ-line cells. Similar granules have been found in the germ lines of insects (Mahowald, 1971) and amphibia (Kalt, 1973; Kerr and Dixon, 1974).

We would like to thank Janice Nowell of the Electron Microscope Facility of the University of California, Santa Cruz, for her help with the technical details of the ultrastructural work presented in this paper. We also express our appreciation to Dr. John S. Pearse for his careful reading and helpful criticism of the manuscript. This work was supported by Public Health Service postdoctoral fellowship 1 FO2 HD52424-01 and 5 FO2 HD52424-02 to M. Houk and by National Science Foundation Grant PCM 76-14726 to R. Hinegardner.

Summary

1. The genital primordium gonads were examined at several stages during their development in the juvenile sea urchin.

2. At metamorphosis the primordial germ cells are a distinct group of cells in the dorsal mesentery, which also supports the stone canal and axial organs. They spread circumferentially along the aboral body wall to form a ring, the genital rachis, completely encircling the anal region. The gonads form as swellings in each interradius of this ring.

3. While the wall of the genitial primordium prior to rachis formation consists of a single layer of epithelial cells, that of the young gonad has two such layers separated by a fluid-filled space.

4. The primordial germs cells of each stage examined are similar in nuclear morphology, cytoplasmic inclusions, and cell shape and size. All stages have germ-cell-specific fibrillo-granular structures, which we name goniosomes.

5. A second cell type, the forerunner of the accessory cell of the mature gonad, is present in the genital tissue of all stages studied. Cellular inclusions characteristic of the accessory cell, including glycogen granules, lipid-like vesicles, and lysozymal-like vesicles, begin to accumulate in these cells even before gonad formation.

LITERATURE CITED

Akesson, B., 1961. A rapid method for orienting small and brittle objects for sectioning in definite planes. *Ark. Zool.,* **13**: 479–482.

Anderson, T. F., 1951. Techniques for the preparation of three dimensional structure in preparing specimens for the electron microscope. *Trans. N. Y. Acad. Sci.,* **13**: 130–134.

Bal, A. K., F. Jubinville, and G. H. Cousineau, 1969. Nuclear activity during oogenesis in sea urchins. II. Fine structural changes and patterns of RNA synthesis during meiotic prophases of *Arbacia punctulata* oocytes. *Z. Zellforsch. Mikrosk. Anat.,* **100**: 180–188.

Bal, A. K., F. Jubinville, G. H. Cousineau and S. Inoue, 1968. Origin and fate of the annulate lamellae in *Arbacia punctulata* eggs. *J. Ultrastruct. Res.,* **25**: 15–28.

Blackler, A. W., 1958. Contribution to the study of germ-cells in *Anura. J. Exp. Morphol.,* **6**: 491–503.

Chatlynne, L., 1969. A histological study of oogenesis in the sea urchin, *Strongylocentrotus purpuratus. Biol. Bull.,* **136**: 167–184.

Chatlynne, L., 1972. An ultrastructural study of oogenesis in the sea urchin *Strongylocentrotus purpuratus.* Ph.D. Dissertation, Oregon State University. 141 pp.

Cuenot, L., 1891. Etudes morphologiques sur les Echinodermes. *Arch. Biol.,* **11**: 313–380.

Davis, H. S., 1971. The gonad wall of Echinodermata: a comparative study based on electron microscopy. Masters Thesis, University of California, San Diego. 99 pp.

Delavault, R., 1966. Determinism of sex. Pp. 615–638 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience Publishers, New York.

Delage, Y., and Hérouard, E., 1903, *Traite de Zoologie concrète les Echinoderms.* Schleicher Freres, Paris, 495 pp.

Dietrich, H. F., and A. R. Fontaine, 1975. A decalcification method for ultrastructure of echinoderm tissue. *Stain Technol.,* **50**: 351–354.

Eddy, E. M., 1975. Germ plasm and the differentiation of the germ cell line. Pp. 229–280 in G. H. Bourne and J. F. Danielli, Eds., *International Review of Cytology.* Vol. 43. Academic Press, New York.

Gurr, E., 1965. P. 211 in *Rational Uses of Dyes in Biology.* Williams and Wilkins Co., Baltimore.

Hamann, O., 1887. Beitrage zure Histologie das Echinodermin. *Jena Z. Naturwiss.,* **21**: 87–262.

Hinegardner, R. T., 1969. Growth and development of the laboratory cultured sea urchin. *Biol. Bull.,* **137**: 465–475.

Holland, N. D., and A. C. Giese, 1965. An autoradiographic investigation of the gonads of the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.,* **128**: 241–258.

Kalt, M. R., 1973. Ultrastructural observations on the germ line of *Xenopus laevis. Z. Zellforsch. Mikrosk. Anat.,* **138**: 41–62.

Kawaguti, S., 1965. Electron microscopy on the ovarian wall of the echinoid with special reference to its muscles and nerve plexis. *Biol. J. Okayama Univ.,* **11**: 66–74.

Kerr, J. B., and K. E. Dixon, 1974. An ultrastructural study of germ plasm in spermatogenesis of *Xenopus laevis. J. Embryol. Exp. Morphol.,* **32**: 573–592.

Longo, F. L., and E. Anderson, 1969. Sperm differentiation in the sea urchins *Arbacia punctulata* and *Strongylocentrotus purpuratus. J. Ultrastruc. Res.,* **27**: 486–509.

MacBride, E. W., 1903. The development of *Echinus esculentus,* together with some points in the development of *E. miliaris* and *E. acutus. Philos. Trans. R. Soc. Lond. B. Biol.,* **195**: 285–327.

Mahowald, A. P., 1971. Polar granules of *Drosophila.* II. Ultrastructural changes during early embryogenesis. *J. Exp. Zool.,* **167**: 237–262.

Masuda, R., and J. C. Dan, 1977. Studies on the annual reproductive cycle of the sea urchin and the acid phosphatase activity of relict ova. *Biol. Bull.,* **153**: 577–590.

Miller, R. A., and H. B. Smith, 1931. Observations on the formation of the egg of *Echinodermata lucunter. Carnegie Inst. Wash. Publ.* No. 413: 47–52.

Millonig, G., M. Bosco, and L. Giambertone, 1968. Fine structure analysis of oogenesis in sea urchins. *J. Exp. Zool.,* **169**: 293–314.

Pearse, J. S., 1969a. Reproductive periodicities of Indo-Pacific invertebrates in the Gulf of Suez. I. The echinoids *Prionocidaris baculosa* (Lamarck) and *Lovenia elongata* (Gray). *Bull. Mar. Sci.,* **19**: 323–350.

Pearse, J. S., 1969b. Reproductive periodicities of Indo-Pacific invertebrates in the Gulf of Suez. II. The echinoid *Echinometra mathaei* (de Blainville). *Bull. Mar. Sci.,* **19**: 580–613.

Prouho, H., 1887. Recherches sur le *Dorocidaris papillata* et quelques autre echinoides de la Mediterranee. *Arch. Zool. Exp. Gen.,* **15**: 213–380.

Reynolds, E. S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell. Biol.,* **17**: 208–213.

Russo, A., 1894. Sul sistema genitale e madreporico degli Echinidi regolari. *Boll. Soc. Nat. Napoli Ser. 1,* **8**: 90–107.

Spurr, A. R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.,* **26**: 31–43.

Verhey, C. A., and F. H. Moyer, 1967. Fine structural changes during sea urchin oogenesis. *J. Exp. Zool.,* **164**: 195–207.

Reference: *Biol. Bull.*, **159**: 295–310. (October, 1980)

THE ULTRASTRUCTURE OF COELOMOCYTES OF THE SEA STAR *DERMASTERIAS IMBRICATA*

EDNA S. KANESHIRO AND RICHARD D. KARP

Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221

It has been previously reported that the sea star, *Dermasterias imbricata,* possesses a true adaptative cell-mediated immune response, as reflected by the specific chronic rejection of integumentary allografts (Karp and Hildemann, 1976). The reaction appears to be mediated by infiltration by phagocytic cells. Since the predominant phagocyte in the sea star is the coelomic amoebocyte (Boolootian and Giese, 1958; Endean, 1966), this cell may mediate the graft reaction. This hypothesis is supported by the recent report of Bertheussen (1979) that phagocytic amoebocytes of the sea urchin *Strongylocentrotus droebachiensis* demonstrate *in vitro* cytotoxic reactions against both allogeneic and xenogeneic echinoid cells. Although several excellent studies characterize the coelomocytes of holothurians and echinoids (Hetzel, 1963; Johnson, 1969a, b, c; Chien *et al.*, 1970; Fontaine and Lambert, 1973, 1977; Bertheussen and Seljelid, 1978; Bertheussen, 1979), only sparse information deals with asteroids. To further understand the immunologic capacities of the sea star, *Dermasterias*, and to compare them with those of other animals, we have characterized the ultrastructure of the coeolomic amoebocyte and have reaffirmed its phagocytic role. Ultrastructural observations on Tiedemann's bodies were also made to determine whether or not the cells of this organ participate in phagocytic activities. We experimented on animals stressed by intermittent extraction of coelomic fluid to follow the recovery of coelomocytes, and to determine whether or not this recovery is due to cell division in the circulating coelomic fluid.

MATERIALS AND METHODS

Animals

Specimens of *Dermasterias imbricata* collected off the California coast were purchased from Pacific Biomarine (Venice, CA) and maintained at 15°C in aquaria containing artificial sea water adjusted to a specific gravity of 1.03–1.04 (Instant Ocean, Aquarium Systems, Wickliffe, OH). The animals were fed lake smelt three times per week. Animals exhibited active locomotion and extension of tube feet, indicating good health.

Isolation of coelomocytes

Coelomic fluid (containing coelomocytes) was obtained by inserting a tuberculin syringe with a #27 gauge needle into the interradial area through the aboral surface of the central disc. Before fluid withdrawal, individual animals were weighed on an Ohaus Harvard trip balance after they were drained of excess sea water for 30 sec.

Coelomic fluid was rapidly added to equal volumes of a fixative solution containing 6% glutaraldehyde and 10 mM ethylene bis (oxyethylene-nitrilo) tetra-acetic acid (EGTA) in Ca^{2+}-free artificial sea water (M.B.L. formula; Cavanaugh, 1964) adjusted to pH 7.4. The addition of the Ca^{2+} chelator and use of Ca^{2+}-free sea water minimized aggregation of coelomocytes (Edds, 1977). Coelomocytes were viewed at ×40 magnification on a Zeiss light microscope and their numbers determined with a hemocytometer.

Light microscopy

Coelomic fluid was placed on glass slides and coelomocytes were observed by phase contrast microscopy as smears or hanging drop preparations. Living coelomocytes were stained for lysosomes with 0.1% neutral red. Cells were allowed to adhere to glass slides coated with bovine serum albumin, then dried and treated with Giemsa stain to aid observations on nuclear configurations.

Electron microscopy

Coelomocytes were prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) by fixation in 3% glutaraldehyde in M.B.L.-formula sea water, post-fixation in 1% OsO_4, and dehydration in ethanol. Coelomocytes for SEM were transferred into acetone and placed on glass cover slips which were coated with poly-L-lysine (Sanders *et al.*, 1975). The preparations were subjected to critical point drying (Denton DCP-1, Denton Vacuum Inc., Cherry Hill, NJ), coated with gold (PS-2 sputter coater, International Scientific Instruments, Glen Ellyn, IL), and viewed on a Stereoscan 600 SEM (Cambridge Scientific Instruments, Ossining, NY).

Cells used for TEM were transferred into propylene oxide, infiltrated, and embedded in plastic (Spurr, 1969). Sections were cut with a diamond knife on a Sorvall MT-1 ultratome and stained with uranyl acetate and lead citrate. Thin sections were viewed on an AEI-6B electron microscope operated at 60 kV.

Tiedemann's bodies were removed from animals which had been injected with 1 ml of the fixative solution. Excised organs were then prepared for TEM as described above.

Clearing of bacteria from coelomic fluid

Specimens of a marine, gram-negative short rod with a single polar flagellum, *Aeromonas* sp., UF-B (isolated and characterized by G. G. Holz, Jr., S.U.N.Y., Upstate Medical Center), were grown in an enriched sea water medium (Soldo and Merlin, 1972) at 37°C for 1 day. Bacteria were concentrated by centrifugation at $2500 \times g$ and washed with M.B.L.-formula sea water. Approximately 3.5×10^7 bacteria in 1 ml of sea water were injected into the coelomic cavity of each sea star tested. Coelomic fluid was withdrawn at various time intervals, and a 0.1 ml portion (undiluted or diluted 1:100) was spread on agar plates prepared with the bacterial growth medium. Bacteria numbers were obtained by colony counts following incubation of agar plates at 37°C for 2 days. Another 2 ml portion was prepared for TEM in the glutaraldehyde fixative described above.

RESULTS

Surface of coelomocytes

The phagocytic amoebocyte is the dominant cell type in the coelomic fluid of *Dermasterias imbricata.* Smaller mononuclear, nonflagellated cells have also been observed. The dynamic nature of the living coelomocytes was apparent by phase microscopy of the cells. Petaloid (bladder), lamellipodial, and filopodial forms were observed. The filopodial form was predominant when cells were allowed to adhere to glass slides. Coelomocytes were in petaloid or lamellipodial configurations when cells were observed immediately after coelomic fluid was sampled, or when cells were fixed immediately upon withdrawal of coelomic fluid from the sea stars. Surfaces of living coelomocytes rapidly and constantly changed shapes, forming pseudopodial extensions that quickly collapsed as still others formed.

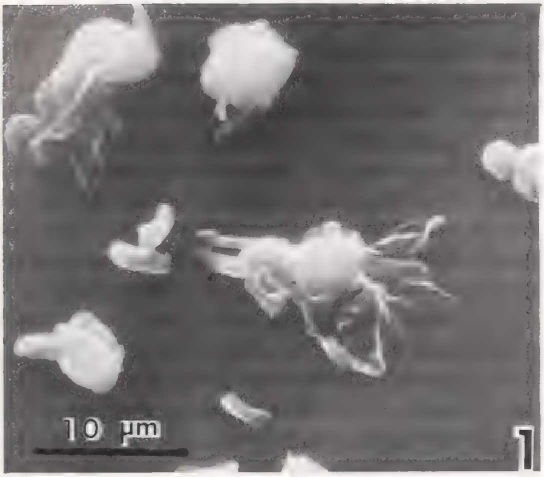
Cells prepared for SEM and TEM were fixed within seconds of withdrawal of coelomic fluid. Observations with SEM (Fig. 1) confirmed light microscopic studies which indicated that the lamellipodial or petaloid configurations were probably characteristic of these cells *in vivo.* Cells from one animal not only had extensive pseudopods, but also had microvilli protruding over the cell surfaces (Fig. 2).

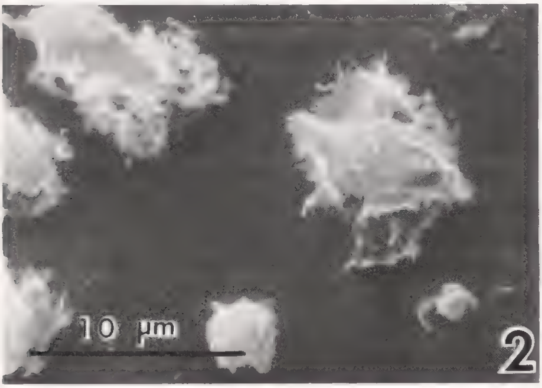
In thin sections viewed by TEM the surface membranes of these cells were greatly folded. Extensions that formed bladders were present in numerous cell profiles (Fig. 3). The extensive folding of the surface made it difficult to distinguish surface membranes from what appeared to be "clear" vesicles.

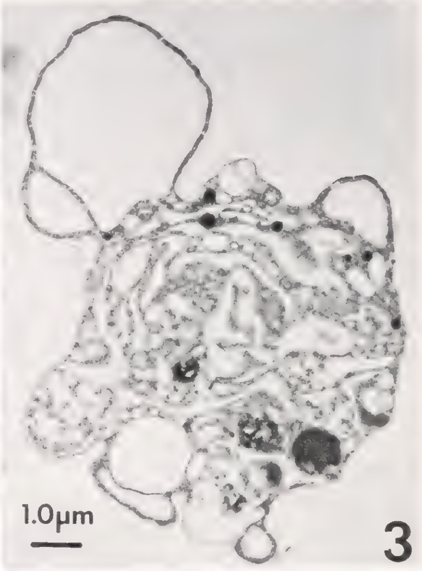
Lysosomes

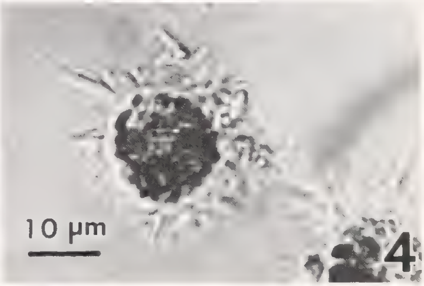
The cytoplasm contained many lysosomal vesicles. Cells stained with neutral red (Fig. 4) and viewed by light microscopy showed large numbers of granules, positive for the stain, concentrated in the central region of the cells. These granules were not observed in pseudopodial extensions. Cells in Figure 4 were photographed after they adhered to the microscope glass slide and hence displayed the filopodial configuration. In TEM the type of vesicles located centrally in coelomocytes, and seen in large numbers within a cell, contained electron-dense material surrounded by less dense material (Fig. 5). These vesicles were of various densities, perhaps reflecting their stage of development. The denser areas within these vesicles .may be regions of condensation or "crystallization" of material packed within the vesicles (Fig. 6).

Another structure, which may be related to a stage in the maturation of these vesicles (or may be an artifact of sample preparation), is shown in Figure 7. These very dense structures often contained a clear area which in turn contained material of even greater density than the main structure. These very dense structures are probably also lysosomes, since such structures are associated with rod-shaped material (Fig. 8) that resembles the material within lysosomal vesicles (*cf.* Fig. 6). Hence, vesicles numerous in the cell and containing dense material surrounded by less dense matrix material are probably the lysosomal neutral-red granules observed by light microscope cytochemical methods. Furthermore, structures resembling the dense rod-shaped material of these vesicles occur in digestive vacuoles (see below) and support our interpretation of the identification and function of these organelles in the cell.









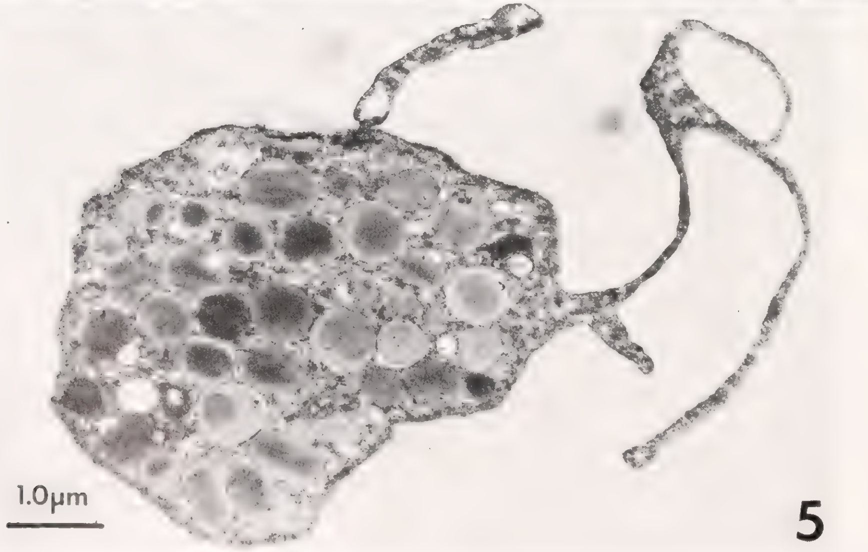


FIGURE 1. Scanning electron micrograph of coelomocytes of *Dermasterias imbricata* showing lamellipodial extensions of the cell surface. × 2000.

Other cell organelles

Where surface extensions formed lamellipods, the protrusions were filled with microfilaments (Fig. 9). Thick linear bundles of filaments in the cytoplasm radiated to the surface of the pseudopod. Organelles such as lysosomes and the nucleus were excluded from these protrusion zones.

The nucleus of coelomocytes was usually bean-shaped or notched, although various profiles were seen because of varying section planes (Fig. 9). Coelomocytes from normal and experimental sea stars were treated with Giemsa stain and observed by light microscopy at various times following removal of coelomic fluid. No nuclear division or mitotic activity was observed.

A Golgi complex was often seen in the notched region of the nucleus as well as in other parts of the cytoplasm. Golgi complexes were generally well developed and had several evenly and closely spaced stacks of lamellae (Fig. 10). Mitochondria were elongate (Fig. 11).

Coelomic fluid samples contained a few cells which had centrioles (basal bodies) associated with striated rootlets (kinetodesmal fibers) (Fig. 12). The combination of these structures suggested that those cells were ciliated or flagellated. These may have been cells normally at other sites but moved during coelomic fluid sampling by a hypodermic needle and syringe. On the other hand these cells may be analogs to the "vibratile" cells described as unique to sea urchins (Johnson, 1969b). *Strongylocentrotus* "vibratile" cells move by a single flagellum in the coelomic fluid. Also uncommon within coelomic fluid samples was a cell with a large vesicle filled with filamentous material (Figs. 13, 14). Again this may not be an *in situ* cell of the coelomic fluid. It is also possible that this cell normally can come from, or go to another tissue such as the Tiedemann's bodies (see below).

Phagocytosis

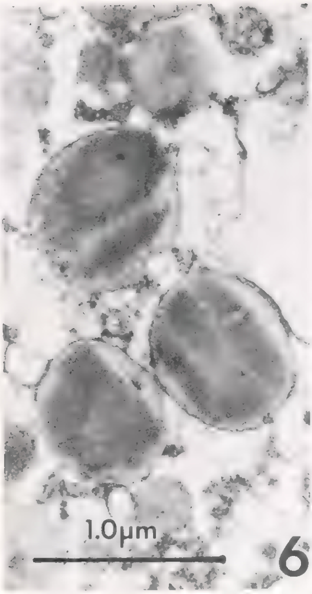
Washed suspensions of a pure culture of a marine *Aeromonas* were injected into sea star coeloms to characterize the phagocytic activity of coelomocytes. Coelomocytes were prepared for TEM at various periods from 5 min to 3 days after injection. By 5 min after the injection, coelomocyte morphology had changed. Large numbers of vacuoles contained structures undergoing digestion (Fig. 15). Bacteria engulfed by pseudopodial activity (Fig. 16) were observed within phagosomes in the cell (Fig. 17). Some bacteria in phagocytic vacuoles were lysed, indicating that digestion had begun (Fig. 18). Vacuoles containing heterogeneous structures were present (Fig. 19). The vacuoles in Figure 19 also contained dense material typical of the lysosomal matrix (see above). Myelin-like figures, indicative of cellular digestive processes, were abundant. These myelin-like figures were present in vesicles (Fig. 20), large vacuoles (Fig. 21),

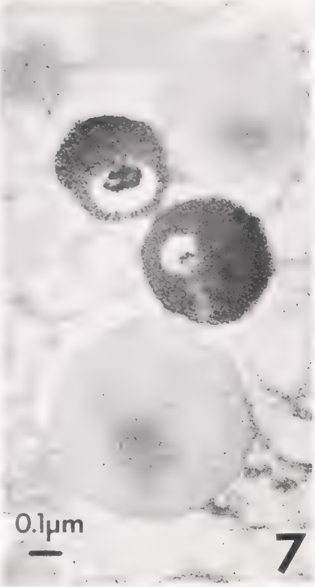
FIGURE 2. *Dermasterias imbricata* coelomocytes with microvilli protruding from their surfaces. Most cells in these samples had microvilli. × 2780.

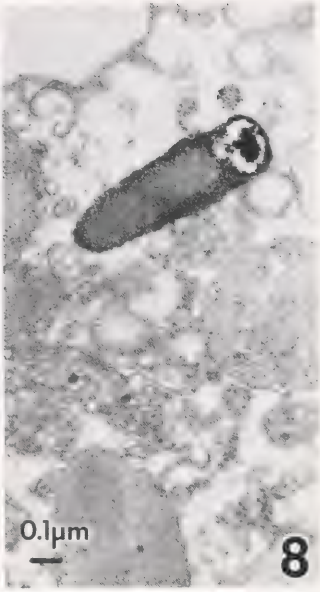
FIGURE 3. Thin section of a coelomocyte, showing the bladder or petaloid surface morphology. Note the highly folded surface membrane and numerous cytoplasmic vesicles. × 6750.

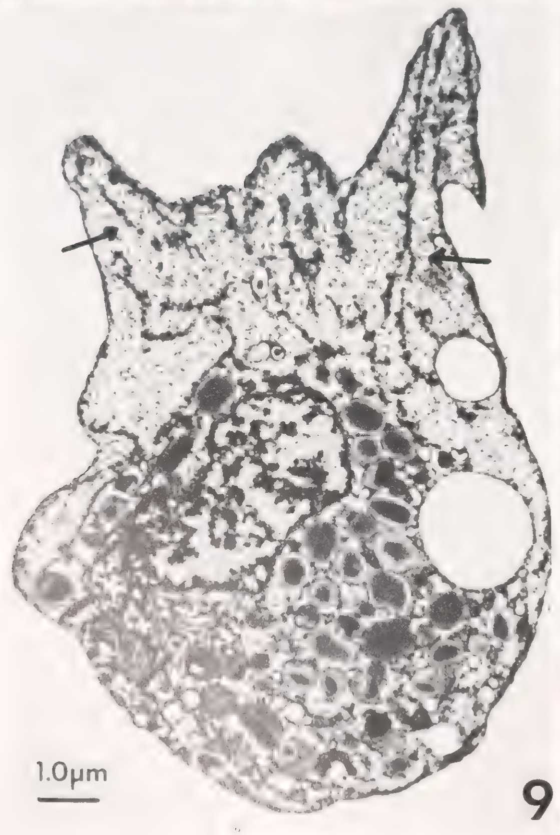
FIGURE 4. Light micrograph of a coelomocyte stained for lysosomes with neutral red. The cell had adhered to the microscope glass slide and thus exhibits filopodial surface extensions. Neutral red-stained granules are restricted to central regions of the cell and do not extend into filopods. × 920.

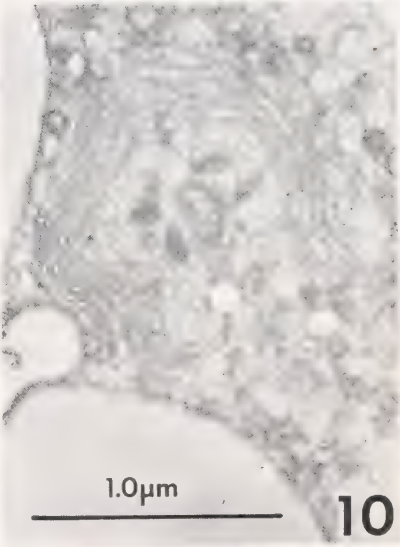
FIGURE 5. Section through a coelomocyte showing high concentrations of lysosomal vesicles. × 13,390.











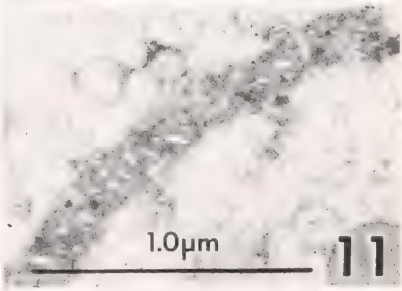


FIGURE 6. Lysosomal vesicles containing more condensed or electron-dense material surrounded by less dense matrices. Denser material often appears in rod-like profiles. This cell was taken from an animal 60 min after bacteria were injected into the coelom. Lysosomal vesicles are similar in cells of untreated animals. × 24,700.

FIGURE 7. Dense vesicles containing clear regions in the cytoplasm. The clear regions contain very dense material (*cf.* more typical lysosomal vesicles next to them and in Fig. 6).

and cell cytoplasm. In many cell profiles it was not possible to determine whether structures that appeared to be related to digestion were distinct phagosomes (secondary lysosomes) or autophagous structures. We did not determine whether coelomocytes self-destruct after they perform their phagocytic function, recover and remain in the coelomic fluid, or are removed from the coelomic fluid.

Aeromonas introduced into the coelomic fluid of sea stars by direct injection of a bacterial suspension were effectively cleared from the coelomic fluid within 2–3 days. No bacterial colonies formed when 0.1 ml of coelomic fluid, withdrawn before injection of bacteria, was plated. This indicates that the coelomic fluid is normally aseptic. Figure *22* shows the rate of disappearance of bacteria from the coelomic fluid of two animals. Also shown is the concentration of coelomocytes in these animals over this time period.

Relationship with the Tiedemann's bodies

Tiedemann's bodies may serve as sites from which coelomocytes arise, through which coelomocytes pass, or where coelomocytes can associate. Our studies do not establish coelomocyte genesis but do suggest a possible relationship and some similarities between cells of the organ and circulating coelomocytes. Tiedemann's bodies are located on the inner side of the peristomial ring and are outpockets of the ring canal of the water vascular system. The paired bodies are approximately 2 mm in diameter and are hollow and highly folded. The folds are lined with columnar or squamous epithelial cells (Hyman, 1955). Figure 23 is a section through an infold showing the lining of epithelial cells and a cell (coelomocyte) in the lumen. The animal was not injected with bacteria but clearly contained numerous bacteria that were probably present in the natural environment and/or the laboratory aquarium. Bacteria were identified in phagocytic vacuoles (Fig. 23) and also within vesicles that resembled lysosomes, *i.e.* appeared to be filled with matrix material (Fig. 24). Some profiles of epithelial cells show notched nuclei (Fig. 23) suggesting that these cells have the same nuclear configuration as do nuclei of coelomocytes. A cell (presumably a coelomocyte) within the lumen, which is contiguous with the water vascular system, contained a large vacuole with a structure having filamentous material (Figs. 25, 26) identical to that observed in a cell in the coelomic fluid (see above and Fig. 13). The nature of the filamentous material is not known, but it resembles smooth muscles seen in Tiedemann's bodies. These observations suggest that coelomocytes can migrate from coelomic fluid, which bathes the exterior of Tiedemann's bodies, to the interior of the organ, which is part of the water vascular system.

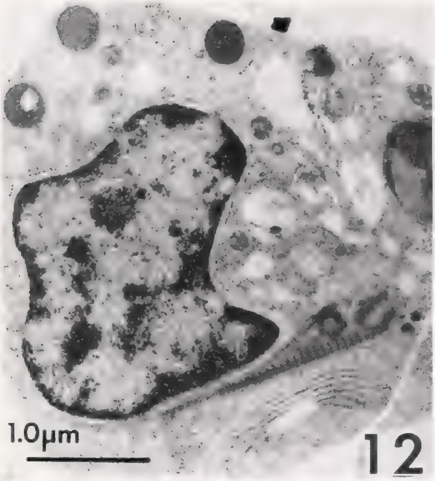
This cell was taken from an animal 5 min after bacteria were injected into the coelomic cavity. These structures are also present in cells of untreated animals. × 39,110.

FIGURE 8. A vesicle with structures resembling those shown in Figures 6 and 7. The rod-shaped element within the very dense matrix suggests these are lysosomes in different stages of maturity. This cell was taken from an animal 5 min after bacteria were injected into the coelom. × 37,660.

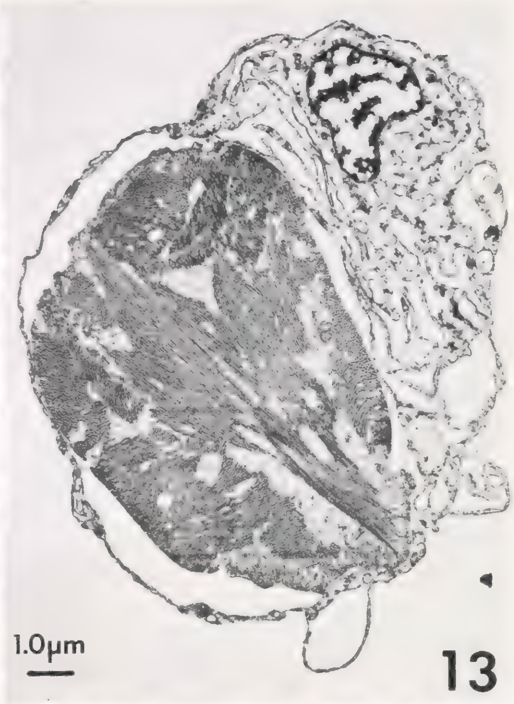
FIGURE 9. A cell with filamentous material in pseudopodial protrusions. Thick bundles (arrows) extend from the central region of the cell to the cell surface. Organelles such as lysosomes and the nucleus are excluded from protrusion zones. This cell was taken from an animal 5 min after bacteria were injected into the coelomic fluid. × 8410.

FIGURE 10. A Golgi apparatus in the cytoplasm of a coelomocyte. Lamellae are even and numerous vesicles are present on the convex mature face of the complex. This cell was taken from an animal 5 min after it was injected with bacteria. Golgi complexes are the same in cells of untreated animals. × 32,880.

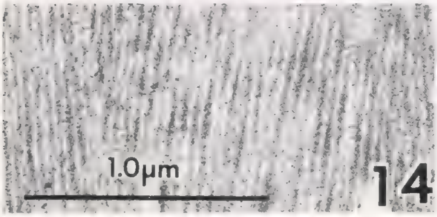
FIGURE 11. A mitochondrion sectioned along its length. The outer membrane and cristae are visible. Electron-dense deposits (also see mitochondria in Fig. 8), probably divalent cation deposits, are present. This cell was taken from an animal 60 min after it was injected with bacteria. Mitochondria have the same morphology in cells of untreated animals. × 37,180.



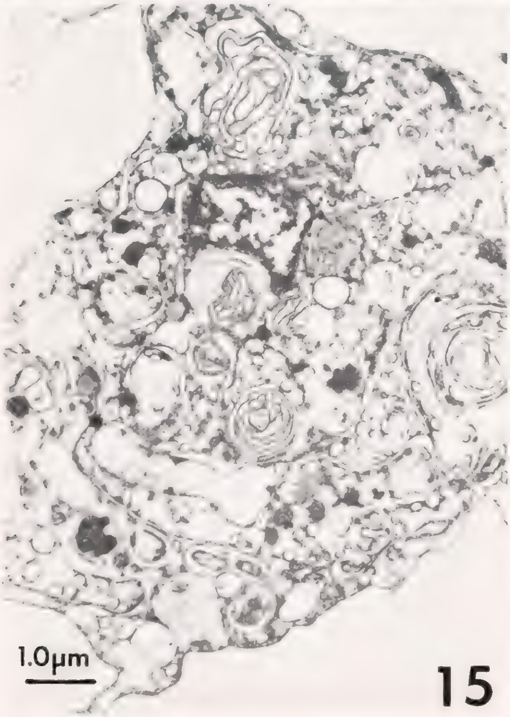
12



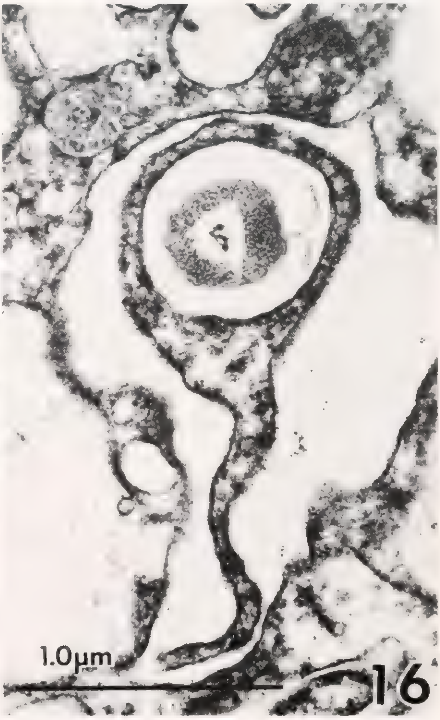
13



14



15



16

FIGURE 12. A cell with basal bodies and striated rootlets, from a coelomic fluid sample. The rootlet appears to associate with the nucleus. ×16,320.

FIGURE 13. A cell with a large vacuole filled with filamentous material from a coelomic fluid sample (*cf.* Figs. 25, 26). The nature of the filamentous material is not known. This cell was taken from a sea star 60 min after the animal was injected with a bacterial suspension. × 6300.

Some cells within the Tiedemann's bodies were flagellated (Fig. 27) and these organelles were associated with elaborate striated rootlets. As described above, we have observed a cell in the coelomic fluid with a basal body and rootlet.

Concentration of coelomocytes in coelomic fluid

Concentration of coelomocytes varied widely from animal to animal. Cell counts ranged from 1.5×10^5/ml to 1.2×10^7 ml in 57 untreated sea stars. There was no obvious correlation of cell number with the animal's size. Similar heterogeneity probably occurs in the natural population. A wide range of percent change in coelomocyte concentration was observed after withdrawal of 7.5 ml (experimental) or 0.5 ml (control) coelomic fluid. Increased concentrations of coelomocytes were observed in 3 out of 6 animals after 1 day; 9 out of 11 after 2 days; 12 out of 15 after 3 days; and 5 out of 10 after 4 days. Some animals did not show any change and a few showed lower coelomocyte concentrations.

Weights of animals taken before withdrawal of 7.5 ml fluid and weights taken at 3 or 4 days after coelomic fluid removal indicated that the animals regained or increased their total body weights. At day 3 there was a mean increase of 5.3 g ($\pm$ 8.6 SD, $N = 18$) and at day 4 a mean increase of 29.5 g ($\pm$ 11.5 SD, $N = 8$).

Discussion

Cell surface

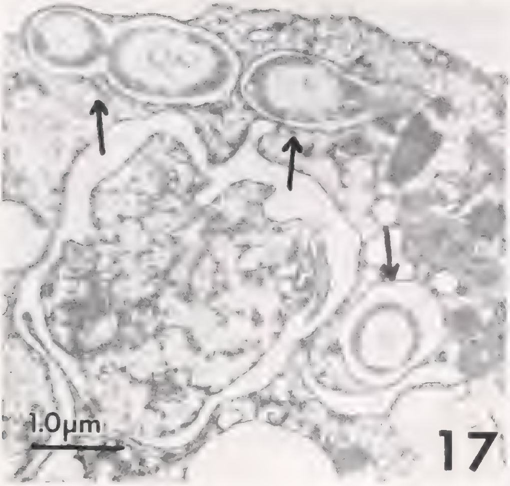
The coelomocytes of the sea star, *Dermasterias imbricata,* have the same general morphology as those reported in echinoids and holothurians (Johnson, 1969a, b, c; Fontaine and Lambert, 1973, 1977). Transformation from lamellipodial or petaloid forms to the filopodial form was observed when cells adhered to glass slides. This transformation has recently been carefully documented in echinoid coelomocytes by Edds (1977) who detected reorganization of microfilament bundles when cells were allowed to settle on glass. The cell shape changes are probably controlled by an actin-myosin-based system, since microfilaments are numerous in pseudopods. Actin has been isolated from echinoid coelomocytes in milligram quantities and its migration in gels characterized (Edds, 1977, 1979; Otto *et al.,* 1979). Echinoid coelomocytes change from petaloid to the lamellipodial forms upon settling on a glass substratum and then to the filopodial form upon hypotonic shock (Otto *et al.,* 1979). The transformation of *Dermasterias* coelomocytes to the filopodial form apparently did not require hypotonic shock, since the change occurred in cells in undiluted coelomic fluid.

Microvilli of various cell types are also known to contain microfilaments and actin-like molecules (Mooseker and Tilney, 1975). Our observations that "villous" structures can appear on surfaces of sea star coelomocytes indicate that these cells have the same capacities as vertebrate macrophages and lymphocytes to produce microvilli (Cohn, 1968). Whether or not smooth *vs.* "villous" surface morphologies were the results of fixation procedures was not examined in this study. The usual "villous" surface morphology of human T- and B-lymphocytes appear

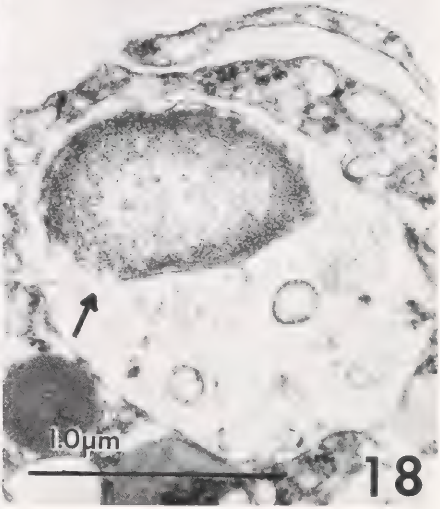
FIGURE 14. A higher magnification of the filamentous material shown in Figure 13. × 31,790.

FIGURE 15. A coelomocyte 60 min after bacteria were injected into the coelomic cavity of the sea water. Bacteria are in vacuoles and digestive processes are indicated by the presence of myelin-like figures, and by numerous vacuoles of mixed contents. ×9100.

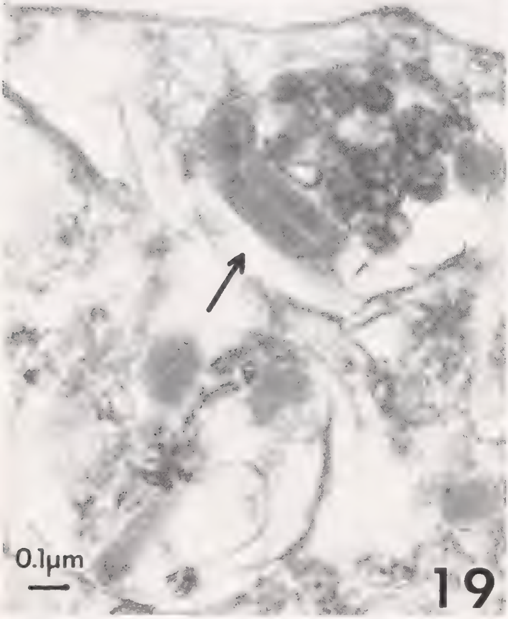
FIGURE 16. A bacterium being "lassoed" by pseudopodial activity of the coelomocyte surface. This sample of coelomic fluid was taken 5 min after bacteria were introduced into the coelom. × 35,200.



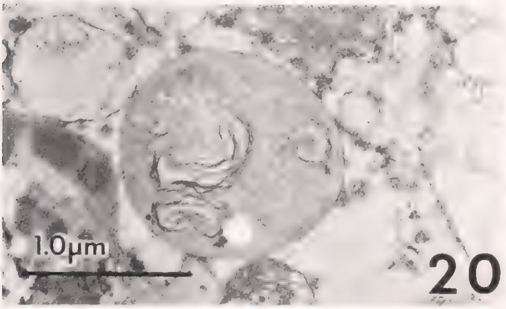
17



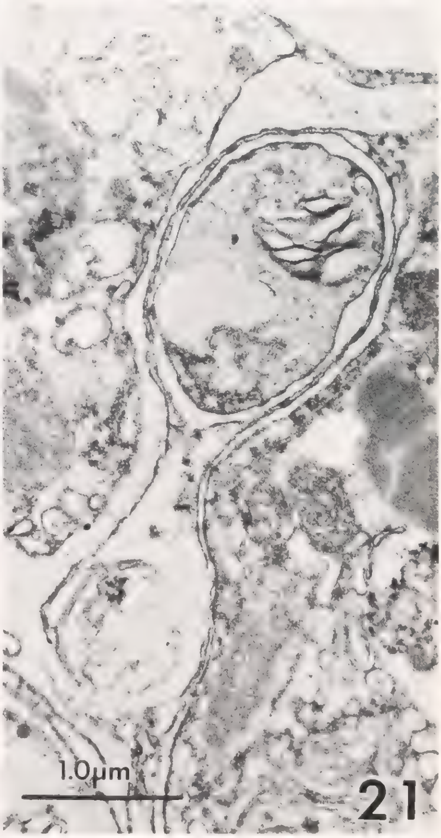
18



19



20



21

FIGURE 17. Several bacteria (arrows) within phagocytic vesicles 5 min after bacteria were introduced into the coelomic cavity of sea stars. × 12,530.

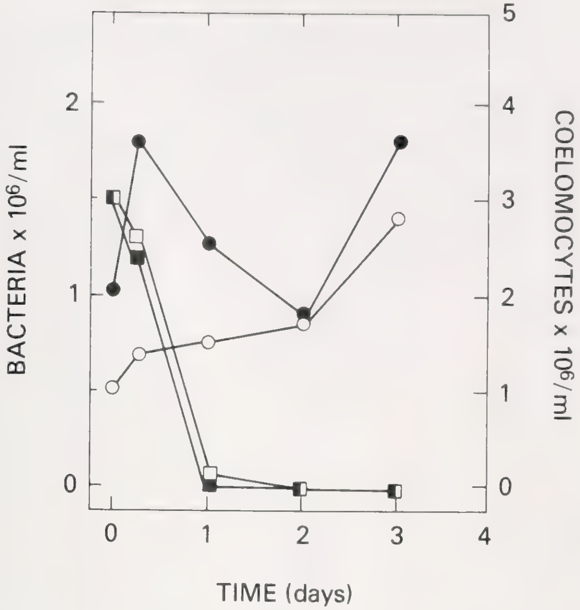


FIGURE 22. The rate of clearing of bacteria from the coelomic fluid of two sea stars (open and closed squares) and concentration of coelomocytes in the two animals (open and closed circles). Animals with bacteria-free coelomic fluid were injected with 3.5×10^7 cells of *Aeromonas* sp.

smooth after various fixation procedures (Alexander *et al.*, 1976). Our observations establish that unlike many cell types, these coelomocytes can exhibit microvilli-covered surfaces when prepared for and viewed by SEM.

Phagocytosis

Numerous studies indicate that echinoderm coelomocytes can actively phagocytize and clear foreign material within a few days after its injection (Bang and Lemma, 1962; Endean, 1966; Johnson, 1969c; Johnson *et al.*, 1970; Reinisch and Bang, 1971; Unkles and Wardlaw, 1976; Wardlaw and Unkles, 1978; Coffaro, 1978). *Dermasterias*, whose normal coelomic fluid was found to be aseptic, also rapidly disposed of injected gram-negative bacteria. Johnson *et al.* (1970) provided ultrastructural evidence that coelomocytes from *Strongylocentrotus* phagocytize bacteria *in vitro* in hanging drops. The cells, however, did not have recognizable primary lysosomes and bacterial cells taken up did not appear

---

FIGURE 18. A bacterium within a phagosome shows signs of being digested. The arrow points to a broken region of the bacterial surface. This cell was taken from the animal 60 min after the animal was injected with bacteria. × 33,860.

FIGURE 19. Large digestive vacuoles are common in the cytoplasm of cells taken 60 min after sea stars were injected with bacteria. The vacuoles contain rod-shaped elements (arrow) similar in density and character to those seen in lysosomes (*cf.* Fig. 6). × 53,210.

FIGURE 20. Vesicles with homogeneous matrix material similar to that seen in lysosomes contained myelin-like figures. This vesicle was in a cell taken 5 min after the animal was injected with bacteria. × 22,160.

FIGURE 21. Vacuoles containing myelin-like figures in a cell taken 5 min after the animal was injected with bacteria. × 21,030.

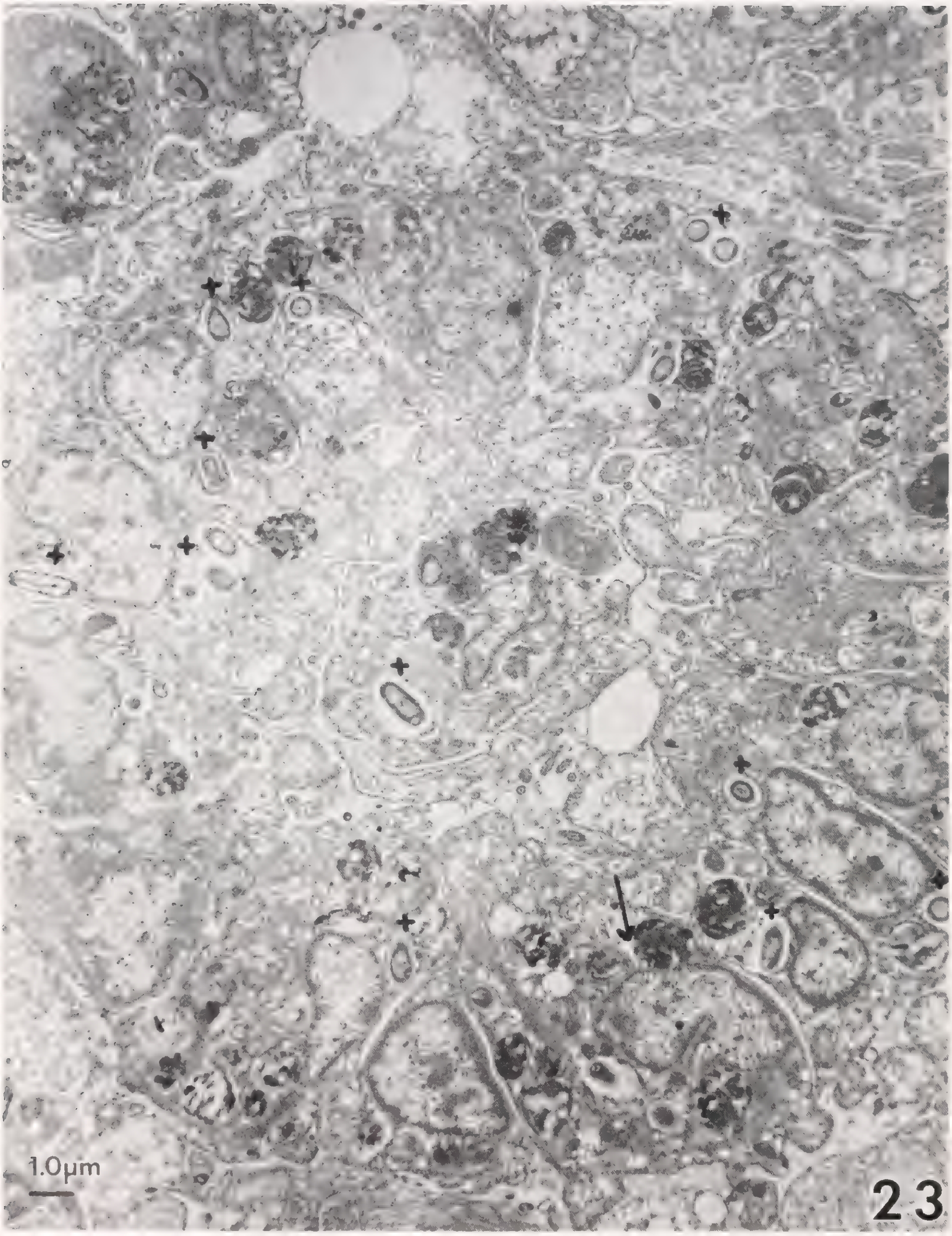
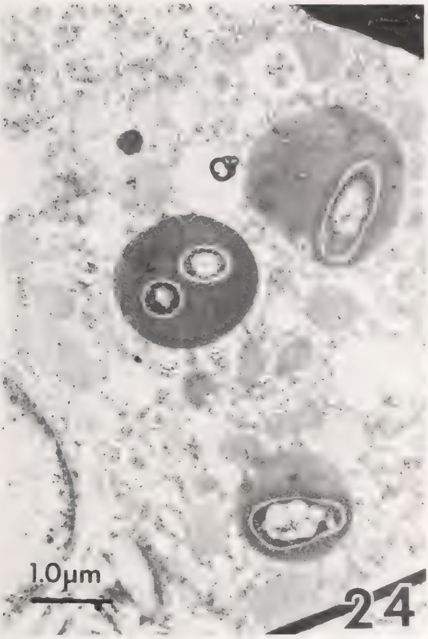
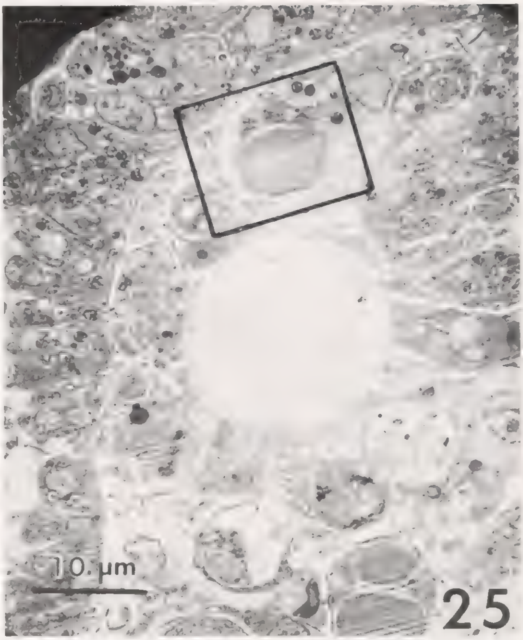
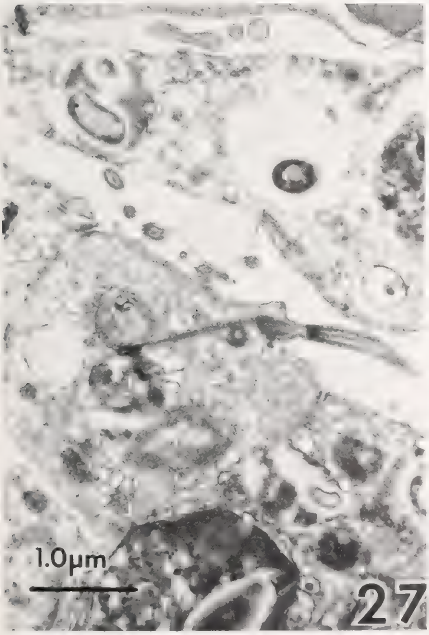


FIGURE 23. A section through a fold within a Tiedemann's body removed from an untreated sea star. Epithelial cells form a circle around a cell in the lumen. The arrow points to an epithelial cell sectioned at a notched region of the nucleus. Crosses indicate bacteria within vesicles in epithelial cells, and within the cell (presumably a coelomocyte) in the lumen. × 6380.

degraded. Several other studies (Bang and Lemma, 1962; Ghiradella, 1965; Endean, 1966; Reinisch and Bang, 1971) have reported that coelomocytes phagocytize foreign matter and then clump within dermal papulae (branchiae). These







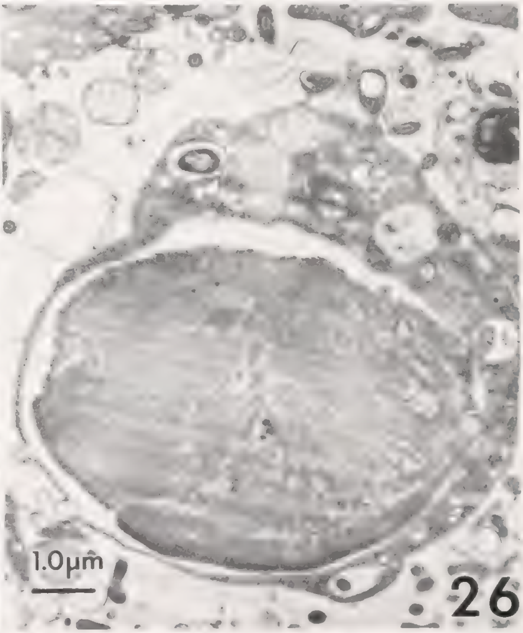


FIGURE 24. Lysosome-like vesicles within a Tiedemann's-body cell. These vesicles with homogeneous matrices contain bacteria. × 10,900.

FIGURE 25. Low magnification of a section through a fold in a Tiedemann's body. The rectangle indicates a cell in the lumen. × 1570.

FIGURE 26. A higher magnification of the cell in the lumen (marked in Fig. 24). This cell has a large vacuole filled with filamentous material (*cf.* Fig. 13). × 8340.

FIGURE 27. A flagellated cell in the Tiedemann's body. The pair of basal bodies and associated striated rootlet are similar to that seen in a cell in a sample of coelomic fluid. × 14,370.

structures then rupture, releasing the coelomocytes to the exterior. Our ultrastructural observations suggest that coelomocytes undergoing extensive phagocytic activity not only are filled with digestive vacuoles but may also participate in autophagous activity. Since the initial bacterial concentration was less than the coelomocyte concentraton, not all digestive vacuoles may have been involved with bacterial elimination. Autophagy in these cells suggests that spent phagocytic coelomocytes of *Dermasterias* self-destruct in the coelomic fluid. Another possibility is that they are expelled from the animal, as observed in other echinoderms (Bang and Lemma, 1962; Endean, 1966; Reinisch and Bang, 1971). It is interesting that the coelomocytes described here show a great deal of similarity to vertebrate macrophages in that they: 1) can produce microvilli on their surfaces, 2) have numerous lysosomes, well developed Golgi complexes (usually located in the nuclear "hof"), notched nuclei, and elongated mitochondria and 3) phagocytize foreign particles forming heterophagic as well as apparent autophagic vacuoles (*cf.* Cohn, 1968).

Tiedemann's bodies

Tiedemann's bodies are organs that may function as primitive lymphoid tissue in sea stars. The organs are outpockets of the water vascular system and are bathed on their outer surfaces by coelomic fluid. Hence, all major fluid systems of the organism circulate around the cells of this organ. The cells of Tiedemann's bodies are capable of endocytosing bacteria. The lumina of the deep folds on the water vascular system side of the organ are filled with cells that resemble coelomocytes (*cf.* Hyman, 1955). The epithelial cells of the Tiedemann's bodies are flagellated, as are those of the radial and ring canals (Hyman, 1955), and possibly serve to move fluid in the water vascular system. Vanden Bossche and Jangoux (1976) have reported that asteroid coelomocytes originate from the coelomic epithelium including the epithelium of Tiedemann's bodies. Since dividing cells were not observed in the coelomic fluid, it appears that circulating coelomocytes of *Dermasterias* might also come from other tissue sources. Animals recovering from extensive withdrawal of coelomic fluid regained total body weights. If that recovery (or increase) in weight reflects coelomic fluid replacement, then the recovery (or increase) in coelomocyte concentrations must result from coelomocyte recruitment. It is possible that coelomocytes originate from epithelial cells, *e.g.* of Tiedemann's bodies. Vanden Bossche and Jangoux (1976) reported that epithelial cells of sea stars became detached and rapidly lost their flagella. The rare cells in coelomic fluid with basal bodies and striated rootlets may represent incompletely transformed recent recruits. The findings reported here reveal indirect evidence that this organ may contribute to the coelomocyte population as well as play an important role in the animal's defense system.

Phagocytic cells participate in rejection of integumentary allografts in *Dermasterias*. The rejection site is heavily infiltrated by large phagocytic cells and smaller mononuclear cells. Autografts do not contain these cell types (Karp and Hildemann, 1976). A number of recent findings implicate the phagocytic amoebocyte as the effector cell. Karp and Johns (1978) reported that coelomic amoebocytes can be stimulated *in vitro* by such mitogenic substances as bacterial lipopolysaccharide and concanavalin A. Bertheussen (1979) reported that the echinoid coelomic phagocyte was able to recognize and react to allogeneic and xenogeneic cells in culture. Other echinoid cell types did not show this activity. Various studies have reported that filopodial coelomocytes participate in "clot" formation and in the elimination of foreign material (Karp and Coffaro, 1980). In addition, preliminary studies indicate that the coelomocyte of *Dermasterias* has surface proteins with molecular weights similar to vertebrate (human and murine) transplantation antigens (L. A. Rheins, J. D. Stinnett, and R. D. Karp, University of Cincinnati, unpublished results). Thus, the phagocytic coelomocyte may mediate specific immune defense reactions in the sea star, and possibly in echinoderms in general. This cell may provide insight into how the sophisticated immune mechanisms of vertebrates evolved.

This work was supported in part by a University of Cincinnati Research Council and Biomedical Research Support awards to E.S.K. and a University of Cincinnati Research Council award and a N.I.A.I.D. research grant (AI 15601) to R.D.K. The authors thank Dr. C. A. Huether and Ms. V. K. Beckham for their assistance in the project and Dr. K. T. Edds for suggestions and comments concerning the manuscript.

SUMMARY

1. The structure of coelomocytes from *Dermasterias imbricata* was characterized by light microscopy and scanning and transmission electron microscopy. The petaloid or lamellipodial configurations were the prevalent forms in freshly drawn coelomic fluid. Cells produced filopodia when they adhered to glass slides.

2. Lysosomes were stained with neutral red and observed by light microscopy, and variations in density and structures of their matrices described by TEM.

3. Animals injected with a bacterial suspension cleared the bacteria from their coelomic fluid within 2–3 days. Ultrastructure of coelomocytes in these animals indicated that phagocytosis and digestion of bacteria were rapid and involved lysosomes.

4. With TEM numerous bacteria were observed within cells of Tiedemann's bodies from untreated animals, indicating that this organ may play an important role in clearing the coelomic fluid and water vascular system of foreign particles.

5. Giemsa-stained coelomocytes of untreated animals and animals recovering from coelomic fluid removal indicated that coelomocytes did not undergo mitosis within the coelomic fluid. Removal of coelomic fluid resulted in recovery or increase in total body weights and coelomocyte concentrations. Hence, circulating coelomocytes must be recruited from other tissue sources in the animal.

LITERATURE CITED

ALEXANDER, E., S. SANDERS, AND R. BRAYLAN, 1976. Purported difference between human T- and B-cell surface morphology is an artifact. *Nature,* **261**: 239–241.

BANG, F. B., AND A. LEMMA, 1962. Bacterial infection and reaction to injury in some echinoderms. *J. Invertebr. Pathol.,* **4**: 401–414.

BERTHEUSSEN, K., 1979. The cytotoxic reaction in allogeneic mixtures of echinoid phagocytes. *Exp. Cell Res.,* **120**: 373–381.

BERTHEUSSEN, K., AND R. SELJELID, 1978. Echinoid phagocytes *in vitro*. *Exp. Cell Res.,* **111**: 401–412.

BOOLOOTIAN, R. A., AND A. C. GIESE, 1958. Coelomic corpuscles of echinoderms. *Biol. Bull.,* **115**: 53–63.

CAVANAUGH, G. M., Ed., 1964. *Marine Biological Laboratory Formulae and Methods V.* Mar. Biol. Lab., Woods Hole, Massachusetts. P. 52.

CHIEN, P. K., P. T. JOHNSON, N. K. HOLLAND, AND F. A. CHAPMAN, 1970. The coelomic elements of sea urchins (*Strongylocentrotus*). IV. Ultrastructure of the coelomocytes. *Protoplasma,* **71**: 419–442.

Coffaro, K. A., 1978. Clearance of bacteriophage T_4 in the sea urchin, *Lytechinus pictus*, *J. Invertebr. Pathol.*, **32**: 384–385.

Cohn, Z. A., 1968. The structure and function of monocytes and macrophages. *Adv. Immunol.*, **9**: 163–214.

Edds, K. T., 1977. Dynamic aspects of filopodia formation by reorganization of microfilaments. *J. Cell Biol.*, **73**: 479–491.

Edds, K. T., 1979. Isolation and characterization of two forms of a cytoskeleton. *J. Cell Biol.*, **83**: 109–115.

Endean, R., 1966. The coelomocytes and coleomic fluids. Pp. 301–328 in R. A. Boolootian, Ed., *Physiology of Echinodermata*, Interscience, New York.

Fontaine, A. R., and P. Lambert, 1973. The fine structure of the haemocyte of the holothurian, *Cucumaria miniata* (Brandt). *Can. J. Zool.*, **51**: 323–332.

Fontaine, A. R., and P. Lambert, 1977. The fine structure of the leucocytes of the holothurian, *Cucumaria miniata*. *Can. J. Zool.*, **55**: 1530–1544.

Ghiradella, H. T., 1965. The reaction of two starfishes, *Patiria miniata* and *Asterias forbesi*, to foreign tissue in the coelom. *Biol. Bull.*, **128**: 77–89.

Hetzel, H. R., 1963. Studies on holothurian coelomocytes. I. A survey of coelomocyte types. *Biol. Bull.*, **125**: 289–301.

Hyman, L. H., 1955. *The Invertebrates: Echinodermata, the Coelomate Bilateria*, Vol. IV. McGraw-Hill, New York. Pp. 274–283.

Johnson, P. T., 1969a. The coelomic elements of sea urchins (*Strongylocentrotus*). I. The normal coelomocytes, their morphology and dynamics in hanging drops. *J. Invertebr. Pathol.*, **13**: 25–41.

Johnson, P. T., 1969b. The coelomic elements of sea urchins (*Strongylocentrotus*). II. Cytochemistry of the coelomocytes. *Histochemie*, **17**: 213–231.

Johnson, P. T., 1969c. The coelomic elements of sea urchins (*Strongylocentrotus*). III. *In vitro* reaction to bacteria. *J. Invertebr. Pathol.*, **13**: 42–62.

Johnson, P. T., P. K. Chien, and F. A. Chapman, 1970. The coelomic elements of sea urchins (*Strongylocentrotus*). V. Ultrastructure of leucocytes exposed to bacteria. *J. Invertebr. Pathol.*, **16**: 466–469.

Karp, R. D., and W. H. Hildemann, 1976. Specific allograft reactivity in the sea star, *Dermasterias imbricata*. *Transplantation*, **22**: 434–439.

Karp, R. D., and J. D. Johns, 1978. Evolution of immune reactivity: Mitogenic responsiveness in the sea star, *Dermasterias imbricata*. *78th Annual Meeting, Am. Soc. Microbiol.*, p. 48.

Karp, R. D., and K. A. Coffaro, 1980. Cellular defense systems in the Echinodermata. In N. Cohen and M. M. Sigel, Eds., *The Reticuloendothelial System: A Comprehensive Treatise*, Vol. V. Plenum Press, New York. In press.

Mooseker, M. S., and L. G. Tilney, 1975. The organization of an actin filament-membrane complex: Filament polarity and membrane attachment in the microvilli of intestinal epithelial cells. *J. Cell Biol.*, **67**: 725–743.

Otto, J. J., R. E. Kane, and J. Bryan, 1979. Formation of filopodia in coelomocytes: Localization of fascin, a 58,000 dalton actin cross-linking protein. *Cell*, **17**: 285–293.

Reinisch, C. L., and F. B. Bang, 1971. Cell recognition reactions of the sea star (*Asterias vulgaris*) to the injection of amebocytes of the sea urchin (*Arbacia punctulata*). *Cell. Immunol.*, **2**: 496–503.

Sanders, S. K., E. L. Alexander, and R. C. Braylan, 1975. A high yield technique for preparing cells fixed in suspension for scanning electron microscopy. *J. Cell Biol.*, **67**: 476–480.

Soldo, A. T., and E. J. Merlin, 1972. The cultivation of symbiote-free marine ciliates in axenic medium. *J. Protozool.*, **19**: 519–524.

Spurr, A. R., 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.*, **26**: 31–43.

Unkles, S. E., and A. C. Wardlaw, 1976. Antibacterial activity in the sea urchin, *Echinus esculentus*. *Soc. Gen. Microbiol. Proc.*, **3**: 182.

Vanden, Bossche, J. P., and M. Jangoux, 1976. Epithelial origin of starfish coelomocytes *Nature*, **261**: 227–228.

Wardlaw, A. C., and S. E. Unkles, 1978. Bactericidal activity of coelomic fluid from the sea urchin, *Echinus esculentus*. *J. Invertebr. Pathol.*, **32**: 25–34.

Reference: *Biol. Bull.*, **159**: 311–318. (October, 1980)

PHOTOPERIODIC CONTROL OF DIAPAUSE IN THE MARINE CALANOID COPEPOD *LABIDOCERA AESTIVA*[1]

NANCY H. MARCUS

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

It has long been recognized that many marine copepods are seasonally present or absent in the plankton. Fish and Johnson (1937) postulated that such species probably survived as resting eggs on the sea bottom during periods unfavorable for their existence in the plankton. Resting eggs in marine bottom sediments have now been documented for several temperate neritic calanoid copepods and cladocerans (Grice and Gibson, 1975, 1977; Johnson, 1980; Kasahara *et al.*, 1974, 1975; Onbe, 1973, 1978), and it has been shown for some of these species that the maximum abundance of resting eggs in the sediments alternates seasonally with the maximum abundance of individuals in the plankton (Kasahara *et al.*, 1975). However, it has been demonstrated experimentally for only a few species that these resting eggs are in diapause rather than quiescence (Grice and Gibson, 1977; Johnson, 1980; Kasahara and Uye, 1979; Marcus, 1979; Zillioux and Gonzalez, 1972).

Diapause typically necessitates complex changes at the biochemical and physiological levels (see Clutter, 1978). In an environment that fluctuates in a predictable way, the ecological and evolutionary success of species must depend on individuals being able to accurately forecast the changes so that sufficient time is allowed for the requisite adaptive responses prior to the onset of unfavorable conditions. Marcus (1979) demonstrated that the onset of egg diapause in the marine calanoid copepod *Labidocera aestiva* precedes the decline in surface water temperatures by 2 weeks, and suggested that some factor other than temperature alone was important in triggering the onset of dormancy in this species. Data presented in this paper demonstrate that photoperiod significantly affects the induction of diapause in *L. aestiva*.

MATERIALS AND METHODS

Rearing of nauplii, copepodites, and adults

Labidocera aestiva were reared from the first naupliar stage through to reproductive maturity in 19 l glass carboys containing 5-μm filtered sea water. The carboys were mounted at an angle on a frame which rotated at 2 rpm. This rotation served to keep the algal food (see below) in suspension. The entire apparatus was in a walk-in incubator equipped with temperature controls and a 24-hr light: dark cycle, with fluorescent lights providing illumination of 200–300 ft-candles. All developmental stages were fed a mixture of *Gymnodinium nelsoni*, *Gonyaulax polyedra*, *Prorocentrum micans*, and *Peridinium trochoideum*. Dinoflagellates were obtained from Robert Guillard, Woods Hole Oceanographic Institution, Woods Hole, MA, and were subsequently cultured in Fernbach flasks containing Guillard's f/2 media (Guillard, 1972) at 17°C and 12L:12D cycle.

---

[1] Contribution Number 4554 of the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543.

Before the copepods reached reproductive maturity, the contents of each carboy were siphoned weekly through a 63 μm Nitex screen to collect surviving copepods. The vessels were washed, refilled with fresh filtered seawater, and supplied with food. The dinoflagellates were added in equal numerical concentrations to give a final density in the carboy of $7.0–10.0 \times 10^2$ cells/ml. Surviving copepods were returned to each carboy. When egg production began, the number of males and females in each carboy was equalized to a density of 25–30 individuals of each sex by removing adults at random with a wide-mouth pipette from the filtrate. Subsequently the carboys were siphoned every 2–3 days so that eggs could be collected before they hatched. Eggs were removed from the filtrate (retained by the screen) with a micropipette and placed in 5-μm filtered sea water. Adults were returned to the carboy. The weekly schedule for changing the sea water and food remained the same.

Experimental procedures

Carboy populations were initiated with 200 nauplii derived from subitaneous eggs (carboys 7, 8, 9, 12, 14, 15) or chilled resting eggs (carboys 4 and 6) produced either in the laboratory (first generation) or by freshly collected field females. Copepods were reared at 13.5°–15.5°C in five carboys exposed to a short-day photoperiod (8L : 16D), and three carboys exposed to a long-day photoperiod (18L : 6D). Eggs were collected from each carboy every 2–3 days for 8–10 days. At each collection the total numbers of eggs and surviving males and females were determined, and a sample of 100–120 eggs (from each collection day) was placed in glass-fiber-filtered sea water and incubated at 25°C and 12L : 12D to hasten the time to hatching. After 4–5 days the percent hatch was determined, and unhatched eggs were placed in two jars (50 eggs/jar) at each of 5° and 25°C. The eggs were kept at these temperatures a minimum of 40 days. At the end of this interval the 5°C eggs were warmed at 25°C and the percent hatch ascertained. At the same time, the hatch of eggs incubated at 25°C was also determined. In addition, a minimum of 20 adult males and females from each carboy were preserved in 5% buffered formalin and subsequently measured with a stereomicroscope to determine cephalothorax and total body length. The body sizes of these copepods and adults collected in the field under similar temperature conditions were compared, to assess the suitability of growth conditions in the laboratory.

RESULTS

The age at reproductive maturity of *Labidocera aestiva* reared in the laboratory at 13.5°–15.5°C ranged from 27.2 ± 1.3 days at 8L : 16D to 22.3 ± 1.5 days at 18L : 6D. The survival of individuals to reproductive adulthood was good (65–75%) for each experimental carboy. Copepods reared under the long-day regime of 18L : 6D produced more eggs during the experimental period (800–1000 eggs in each carboy per day) than the individuals reared under the short-day regime of 8L : 16D (227–580 eggs in each carboy per day).

Table I shows the percent hatch data for one set of eggs for each collection day for carboy 6, and is representative of results for the other egg set as well as the other carboys. For carboy 6, final hatch at 25°C was high (> 90%) after chilling at 5°C for incubation periods ranging from 49 to 123 days. The hatch of eggs kept at 25°C appears to increase (26–43%) as the incubation period

TABLE I

Percent hatch of eggs produced by females reared at 13.5°–15.5°C and 8L:16D in Carboy 6. Eggs that did not hatch within 4–5 days at 25°C (initial) were incubated in jars at 5° or 25°C. The final hatch of these eggs at 25°C is indicated, as well as the period of incubation.

Date collected	Initial hatch at 25°C	Incubation (days)	Final hatch	
			5°C	25°C
3 August 1979	4%	49	95%	26%
6 August 1979	1%	62	97%	36%
8 August 1979	7%	81	90%	39%
10 August 1979	2%	99	93%	37%
13 August 1979	7%	123	91%	43%

increases. The percent hatch of eggs and adult body lengths for each carboy is shown in Table II. The values for percent initial hatch for each carboy represent an average of values determined each time the eggs were collected. Similarly, the values for percent final hatch for each carboy after incubation at 5° and 25°C represent an average of values derived from the unhatched eggs subsequently incubated from each collection day.

Subitaneous (quickly hatching) and diapause egg production were strikingly different between the two photoperiod regimes. At 8L:16D, 13.2 ± 4.3% of the eggs produced hatched within 4–5 days (classified as subitaneous), whereas at 18L:6D, 89.9 ± 1.4% of the eggs were subitaneous. The similarity of subitaneous egg production among replicates was slightly less at 8L:16D than at 18L:6D, ranging from 2.9 ± 0.8% to 23.8% ± 1.9, and 87.3 ± 2.5% to 92.0 ± 1.7%, respectively. Eggs produced at 18L:6D that did not hatch within 4–5 days appeared brown and granular. They were dead eggs (Marcus, unpublished), and were not incubated at 5° or 25°C. Most of the unhatched eggs produced at 8L:16D were green and had a clear perimeter. These eggs were incubated at 5° and 25°C and, as shown in Table II, an average of 85.9 ± 3.1% hatched after chilling and were classified as diapause eggs. The remainder, which did not hatch, were dead. Some of the eggs (38.1 ± 5.3%) kept at 25°C did hatch during the incubation interval.

The difference in body lengths of adult males and females reared in replicate carboys for a given photoperiod regime was small. The widest range of values resulted at 8L:16D for male total length (2.10 ± 0.11 mm to 2.24 ± 0.13 mm). For each experimental regime females were longer than males (both total length and cephalothorax). Moreover, individuals reared under the short-day regime (8L:16D) were larger than individuals reared at 18L:6D.

DISCUSSION

The results presented in this paper provide the first evidence of the importance of photoperiod as a trigger for the induction of diapause in a marine copeopd, although this effect has been shown for several fresh-water copepods and cladocerans (Stross and Hill, 1965; Stross, 1969a, b; Watson and Smallman, 1971; Bunner and Halcrow, 1977). Copepods which developed at 13.5°–15.5°C under 18L:6D produced 89.9 ± 1.4% subitaneous eggs, whereas individuals reared at 8L:16D produced only 13.2 ± 4.3% subitaneous eggs. Moreover, 85.9 ± 3.1% of the non-subitaneous eggs produced at 8L:16D hatched synchronously at 25°C after

TABLE II

Percent hatch of eggs produced by females reared at 13.5°–15.5°C under 8L:16D or 18L:6D. Eggs that did not hatch within 4–5 days at 25°C (initial) were incubated in jars at 5° and 25°C. The final hatch of these eggs at 25°C is indicated, as well as the cephalothorax (CT) and total body (TL) lengths in millimeters attained by adult males and females for each carboy. $\bar{X}$ = mean.

Photoperiod	Carboy #	Percent hatch (±SE)			Size (±SD)			
		Initial hatch at 25°C	Final hatch		♀		♂	
			5°C	25°C	CT	TL	CT	TL
8L:16D	4	13.1 ± 3.3	82.4 ± 5.8	23.0 ± 5.2	1.74 ± 0.07	2.37 ± 0.09	1.72 ± 0.07	2.24 ± 0.13
	6	4.2 ± 1.2	92.2 ± 1.1	34.7 ± 3.4	1.80 ± 0.07	2.40 ± 0.10	1.70 ± 0.05	2.23 ± 0.08
	7	22.0 ± 4.1	89.3 ± 1.8	34.1 ± 4.1	1.78 ± 0.08	2.37 ± 0.09	1.71 ± 0.09	2.22 ± 0.10
	8	2.9 ± 0.8	89.9 ± 1.8	44.4 ± 6.4	1.70 ± 0.05	2.26 ± 0.08	1.65 ± 0.06	2.14 ± 0.08
	9	23.8 ± 1.9	75.5 ± 5.1	54.4 ± 5.77	1.72 ± 0.06	2.28 ± 0.08	1.61 ± 0.07	2.10 ± 0.11
$\bar{X}$		13.2 ± 4.3	85.9 ± 3.1	38.1 ± 5.3	1.75 ± 0.04	2.23 ± 0.06	1.68 ± 0.05	2.19 ± 0.06
18L:16D	12	87.3 ± 2.5	—	—	1.66 ± 0.08	2.21 ± 0.12	1.55 ± 0.08	2.03 ± 0.10
	14	90.4 ± 1.8	—	—	1.66 ± 0.05	2.21 ± 0.09	1.57 ± 0.05	2.07 ± 0.06
	15	92.0 ± 1.7	—	—	1.70 ± 0.06	2.24 ± 0.07	1.58 ± 0.06	2.08 ± 0.06
$\bar{X}$		89.9 ± 1.4			1.67 ± 0.02	2.22 ± 0.02	1.57 ± 0.02	2.06 ± 0.03

chilling at 5°C. However, chilling was not an absolute requirement to induce hatching, since some eggs kept at 25°C for a prolonged period did hatch (Tables I, II). The hatching response of diapause eggs produced in the laboratory under a short-day regime is similar to the pattern demonstrated for diapause eggs produced by field-collected females (Marcus, 1979). Copepods reared in the laboratory under a long-day regime did not produce diapause eggs.

The genetic composition of a female ultimately restricts the potential range of egg types she can produce, but the egg type actually realized is determined by the environmental conditions she experiences during development. This is evident from the production of diapause eggs at 8L : 16D by females which developed from both diapause eggs (carboys 4 and 6) and subitaneous eggs (carboys 7, 8, and 9). Similarly, subitaneous eggs were produced at 18L : 6D by females which developed from subitaneous eggs (carboys 12, 14, and 15).

The data on body sizes of *L. aestiva* reported herein provide a valid estimate (see Paffenhöffer, 1970) of the suitability of the laboratory conditions tested in this study. The total lengths and cephalothorax lengths attained by adult males and females reared at 13.5°–15.5°C under both long- and short-day regimes (Table II) are comparable to the sizes of field adults collected at a similar water temperature in Vineyard Sound, MA (Marcus, 1979). The fact that individuals reared at 18L : 6D were smaller than individuals reared at 8L : 16D was probably due to temperature. The incubator in which the copepods were reared undergoes a slight temperature change as a result of the light cycle. When the lights were on, the air temperature in the incubator was maintained at 15.5°C, but with the lights off the temperature dropped to 13.5°C. Presumably the water in the carboys also experienced this temperature shift, although to a lesser extent. Thus, the copepods reared under the 18L : 6D regime experience slightly warmer temperatures overall (average 15.0°C/L : D cycle), than individuals reared at 8L : 16D (average 14.2°C/L : D cycle). Based upon the inverse relationship between body size and temperature which has been documented for *Labidocera aestiva* collected in the field (Marcus, 1979), the differences observed for the laboratory-reared copepods can perhaps be accounted for by the slightly different thermal regimes.

An alternative explanation, based on feeding patterns, could also account for the observed differences. If *L. aestiva* feeds primarily at night, it is possible that under the short-day regime (8L : 16D) individuals were able to consume more food and therefore attained a larger size. If this were the case, however, it might be expected that greater food consumption would result in an increase in the number of eggs produced. These results were not obtained. In fact, copepods reared at 8L : 16D produced fewer eggs. It may be that a diapause egg is able to overwinter because it contains more storage nutrients than a subitaneous egg. This could result in the production of fewer diapause eggs to balance the increased energy requirement. Without more information on *L. aestiva* feeding pattern and egg biochemistry, this problem cannot be clarified. Nevertheless, sizes attained by laboratory animals suggest that conditions used to rear *L. aestiva* in the laboratory adequately simulated the conditions for good development, growth, and reproduction experienced by this species in the field. Therefore, it is assumed that the factors which were shown to stimulate the production of diapause and subitaneous eggs in the laboratory reflect a similar function in the field.

The influence of photoperiod on the life history of *L. aestiva*, as suggested by this study, correlates well with the annual life cycle of this species in Vineyard Sound, MA. (Fig. 1). *L. aestiva* adults first appear in the plankton in early

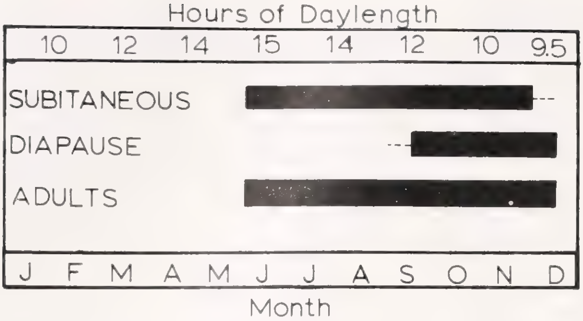


FIGURE 1. Schematic diagram of seasonal occurrence of adults and production of subitaneous and diapause eggs of *Labidocera aestiva* in Vineyard Sound, MA, with respect to daylength.

summer when surface water temperatures have reached 18°–20°C (Marcus, 1979) and the photoperiod is 15L:9D (U. S. Dept. of Commerce, 1979). In August, daylength is somewhat shorter (14L), and water temperature is maximal (22°–23°C). By mid-September, surface water temperature has declined to 19°C and photoperiod is 12L:12D. Temperatures drop to 15°C by mid-November, and daylength (9 3/4L) is further reduced. In mid-December when nauplii, copepodites, and adults disappear from the plankton, surface water temperature has dropped to 6°C, and daylength is minimal at 9 1/2L:14 1/2D. *Labidocera aestiva* produces both subitaneous and diapause eggs in November in Vineyard Sound (Marcus, 1979). Similarly, *L. aestiva* reared in the laboratory under the regime (13.5°–15.5°C, 8L:16D) that approximates Vineyard Sound in November produce both subitaneous and diapause eggs. Moreover, the majority of eggs produced by this combination of photoperiod and temperature in both the field and laboratory are diapause eggs. The same combination of factors again prevail in Vineyard Sound in mid-March, but at this time *L. aestiva* adults are not present. When they do reappear in early summer the photoperiod is approximately 15L:9D, and only subitaneous eggs are produced. The results for the alternate regime (13.5°–15.5°C, 18L:6D) tested in this study, while not exactly identical to the field situation experienced by *L. aestiva*, nevertheless suggest that under long-day photoperiods, this species produces subitaneous eggs. The results do not imply that photoperiod is the only factor influencing the production of diapause eggs by *L. aestiva*, nor that it is of primary importance. Other factors (*e.g.*, temperature, density, food) may have a similar function and/or may modify the effects of photoperiod, as has been shown for insects (Saunders, 1976), freshwater copepods (Watson and Smallman, 1971), and cladocerans (Bunner and Halcrow, 1977; Stross, 1969b; Stross and Hill, 1965). Thus, it is possible that changes in the quantity or quality of the food supply during the weekly feeding interval may have influenced the type of eggs produced by *L. aestiva* in this study. Most recently, it has been suggested that temperatures below 15°C induce the production of dormant eggs by the estuarine calanoid copepod, *Acartia californiensis* (Johnson, 1980). Gaining insight into the factors which control diapause of marine copepods is fundamental to elucidating the mechanism that regulates their seasonal occurrence in the plankton.

I thank George Grice and Timothy Cowles for their helpful comments on the manuscript, and Carol Riley for her assistance with the research. This work was supported by NSF Grant OCE78-08857.

Summary

The calanoid copepod *Labidocera aestiva* was reared in the laboratory at 15°C. Individuals that developed under a photoperiod regime of 18L:6D produced subitaneous eggs, whereas copepods exposed to a short-day regime of 8L:16D produced mostly diapause eggs. The results indicate that photoperiod is an important factor controlling the life cycle of *L. aestiva*. It is suggested that in Vineyard Sound, MA, this species produces subitaneous eggs during the summer in response to long daylengths, and in the fall produces mostly diapause eggs in response to short daylengths.

LITERATURE CITED

Bunner, H. C., and K. Halcrow, 1977. Experimental induction of the production of ephippia by *Daphnia magna* Straus (Cladocera). *Crustaceana,* **32**: 77–86.

Clutter, M., 1978 (ed.) *Dormancy and Developmental Arrest.* Academic Press, New York, 316 pp.

Fish, C., and M. Johnson, 1937. The biology of zooplankton populations in the Bay of Fundy and Gulf of Maine with special reference to production and distribution. *J. Fish. Res. Bd. Can.,* **3**: 189–233.

Grice, G., and V. Gibson, 1975. Occurrence, variability, and significance of resting eggs of the calanoid copepod, *Labidocera aestiva. Mar. Biol.,* **31**: 335–357.

Grice, G., and V. Gibson, 1977. Resting eggs in *Pontella meadi* (Copepoda: Calanoida). *J. Fish. Res. Bd. Can.,* **34**: 410–412.

Grice, G., and T. Lawson, 1976. Resting eggs in the marine calanoid copepod, *Labidocera aestiva* Wheeler. *Crustaceana,* **30**: 9–12.

Guillard, R., 1972. Culture of phytoplankton for feeding marine invertebrates. Pp. 29–60 in W. Smith and M. Chanley, Eds., *Culture of Marine Invertebrate Animals,* Plenum Press, New York.

Johnson, J., 1980. Effects of temperature and salinity on production and hatching of dormant eggs of *Acartia californiensis* (Copepoda) in an Oregon estuary. *Fishery Bulletin, Canada,* **77**: 567–584.

Kasahara, S., and S. Uye, 1979. Calanoid copepod eggs in sea-bottom muds. V. Seasonal changes in hatching of subitaneous and diapause eggs of *Tortanus forcipatus. Mar. Biol.,* **55**: 63–68.

Kasahara, S., S. Uye, and T. Onbe, 1974. Calanoid copepod eggs in sea-bottom muds. *Mar. Biol.,* **26**: 167–171.

Kasahara, S., S. Uye, and T. Onbe, 1975. Calanoid copepod eggs in sea-bottom muds. II. Seasonal cycles of abundance in the populations of several species of copepods and their eggs in the Inland Sea of Japan. *Mar. Biol.,* **31**: 25–29.

Marcus, N., 1979. On the population biology and nature of diapause of *Labidocera aestiva* (Copepoda: Calanoida). *Biol. Bull.,* **157**: 297–305.

Onbe, T., 1973. Preliminary notes on the biology of the resting eggs of marine cladocerans. *Bull. Plankton Soc. Jpn.,* **20**: 74–77.

Onbe, T., 1978. Distribution of the resting eggs of marine cladocerans in the bottom sediment of Ise Bay and Uraganmi Inlet, Central Japan. *Bull. Jpn. Soc. Sci. Fish.,* **44**: 1053.

Paffenhoffer, G., 1970. Cultivation of *Calanus helgolandicus* under controlled conditions. *Helgol. Wiss. Meeresunters.,* **20**: 346–359.

Saunders, D. S., 1976. *Insect Clocks.* Pergamon Press, Oxford. 279 pp.

Stross, R., 1969a. Photoperiod control of diapause in *Daphnia.* II. Induction of winter diapause in the Arctic. *Biol. Bull.,* **136**: 264–273.

Stross, R., 1969b. Photoperiod control of diapause in *Daphnia.* III. Two-stimulus control of long-day, short-day induction. *Biol. Bull.,* **137**: 359–374.

STROSS, R., AND J. C. HILL, 1965. Diapause induction in *Daphnia* requires two stimuli. *Science,* **150**: 1462–1464.

UNITED STATES DEPARTMENT OF COMMERCE, 1979. *Tide Tables, East Coast of North and South America.* U. S. Government Printing Office, Washington, D. C. 293 pp.

WATSON, N., AND B. SMALLMAN, 1971. The role of photoperiod and temperature in the induction and termination of an arrested development in two species of freshwater cyclopoid copepods. *Can. J. Zool.,* **49**: 855–862.

ZILLIOUX, E., AND J. GONZALEZ, 1972. Egg dormancy in the neretic calanoid copepod and its implications to overwintering in boreal waters. *Arch. Limnol. European Marine Biology Symposium,* **5**: 217–230.

Reference: *Biol. Bull.*, **159**: 319–324. (October, 1980)

FURTHER IMPROVEMENT UPON MAINTENANCE OF ADULT SQUID (*DORYTEUTHIS BLEEKERI*) IN A SMALL CIRCULAR AND CLOSED-SYSTEM AQUARIUM TANK

GEN MATSUMOTO AND JUNICHI SHIMADA

Electrotechnical Laboratory, Optoelectronics Section, Tsukuba Science City, Ibaraki 305, Japan

The squid *Loligo, Doryteuthis* and *Sepioteuthis* have large nerve fibers that make them particularly amenable to experimentation (Arnold *et al.*, 1974). However, difficulties in squid experiments result from regional and seasonal limitations on the availability of natural squid stocks. These limitations can be partially eliminated if the squid are maintained in an aquarium in the laboratory (Matsumoto, 1976).

We tried maintenance of adult squid at our laboratory located at Tanashi, a 3–5 hr drive from the fishing site near Jogashima Island, Kanagawa Prefecture (Matsumoto, 1976). We adopted a closed system with a circular tank where sea water was circulated peripherally along the tank wall to mitigate head-on collisions of the squid with the wall. We adopted this approach mainly because of the conclusions of Summers and McMahon (1974), and Summers *et al.*, (1974), that skin damage resulting from collisions with tank walls was a major factor limiting the survival of the squid in a small (*e.g.*, 1.68 m^2) tank. Similar closed seawater systems with tank capacities of 1000 and 10,000 l were used by Hanlon *et al.* (1978) to maintain *Loligo plei* and *Loligo pealei*, with mean survival times of 13–25 days. O'Dor *et al.* (1977) obtained 32–82 day survival of specimens of *Illex illecebrosus* by mitigating collisions using a 15-m-diameter circular tank in an open system. We maintained squid (*Doryteuthis bleekeri*) in our closed system tank for as long as 2 weeks. We concluded that filtering ability was essential to squid survival in our closed system, and that skin damage was minor among causes of mortality in a 2-week maintenance period (Matsumoto, 1976). The question became: Can survival be improved if filtering ability is increased?

MATERIALS AND METHODS

The source of squid (*Doryteuthis bleekeri*), method of transporting them, and the aquarium system used in these experiments was similar to the one described in Matsumoto (1976): It consisted of an inner and an outer circular tank, three filtering stages, a charcoal filter, a dirt trap, a circulation pump, a temperature controller, and an air bubbler (Fig. 1). The outlet was directed so that filtered sea water flowed peripherally along the circular walls. Net patterns were drawn with black vinyl paint on the inner and the outer tank walls (Fig. 2). The peripherally circulating flow and these net patterns were particularly useful in reducing squid collisions with the tank walls (Matsumoto, 1976). Inner and outer tank walls were light brown polyethylene. Their diameters were 1.5 and 0.5 m, respectively, and their depth 1.2 m. Filtering stage 3, the charcoal filter, dirt trap, circulation pump, and temperature controller were the same as in Matsumoto (1976). Filtering stage 1 was composed of layers of commercially available zeolite and sand. The average diameter of zeolite grains was about 3 mm, and the thickness of the layer 15 cm. The

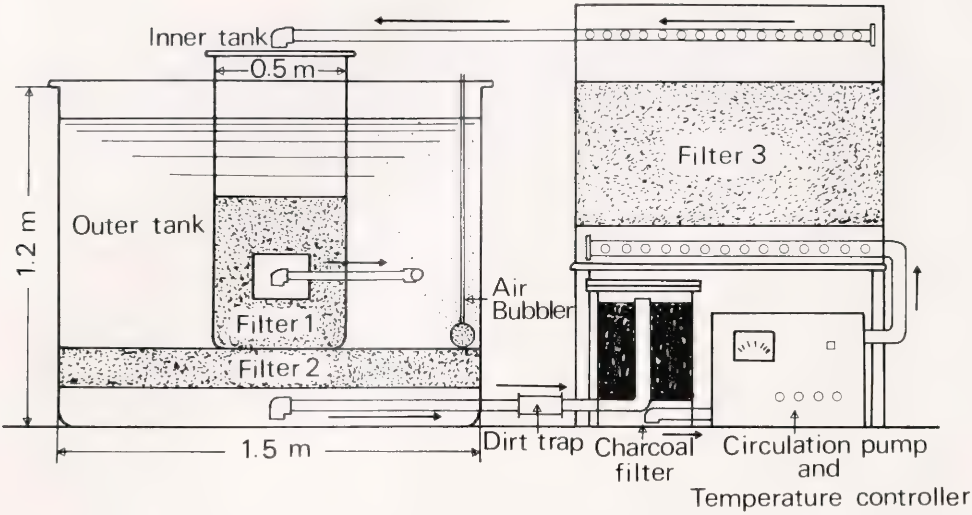


FIGURE 1. Configuration of the aquarium system. The arrows show the sequence of recirculating sea water through the system.

average diameter of the sand grains was 2 mm and the layer's thickness in most runs was 20 cm. The zeolite in the filter weighed 20 kg. In our previous system (Matsumoto, 1976) filter 1 contained a 5-cm layer of zeolite. Filtering stage 2 was the same as described in our previous report, except that 10 kg of crushed oyster shell was used to cover the filter surface. No direct sunlight entered the room when our laboratory was located at Tanashi, Tokyo. After our move to Tsukuba, Ibaraki prefecture, a 6–12 hr drive from the fishing site near Jogashima Island, the aquarium system was roofed to protect it from the rain but direct sunlight reached the system, resulting in the growth of greenish algae on the tank walls. At Tanashi and Tsukuba, a 40 W incandescent light about 1 m above the aquarium was kept lit day and night to help the squid see the tank walls.

Maintenance conditions were the same as those in Matsumoto (1976). Sea water flowed at the rate of 20 l/min through the system, and sea water temperature was kept between 15° and 18°C by a temperature controller. When squid were transferred to the aquarium from a transportation tank, aquarium temperature was adjusted to that in the tank. After transfer the temperature of sea water in the aquarium was lowered at less than 0.5°C/hr to between 15° and 18°C.

RESULTS

Maintenance of unfed squid

In November and December, 1978, we tried three runs of 2-week maintenance of squid (*Doryteuthis bleekeri*) by putting 15, 18, and 20 squid, respectively, into the aquarium without feeding them. The three runs resulted in no natural deaths, but eight squid were killed by cannibalism. No cannibalism was observed during first 3 days after the squid were transported from the fishing site.

Survival was appreciably improved as compared with that in our previous experiment (see the dashed line in Fig. 3). The system and maintenence conditions had not been changed, except for the replacement of the sand by 20 kg zeolite in filter 1 and the addition of oyster shell. The shell alone did not appreciably

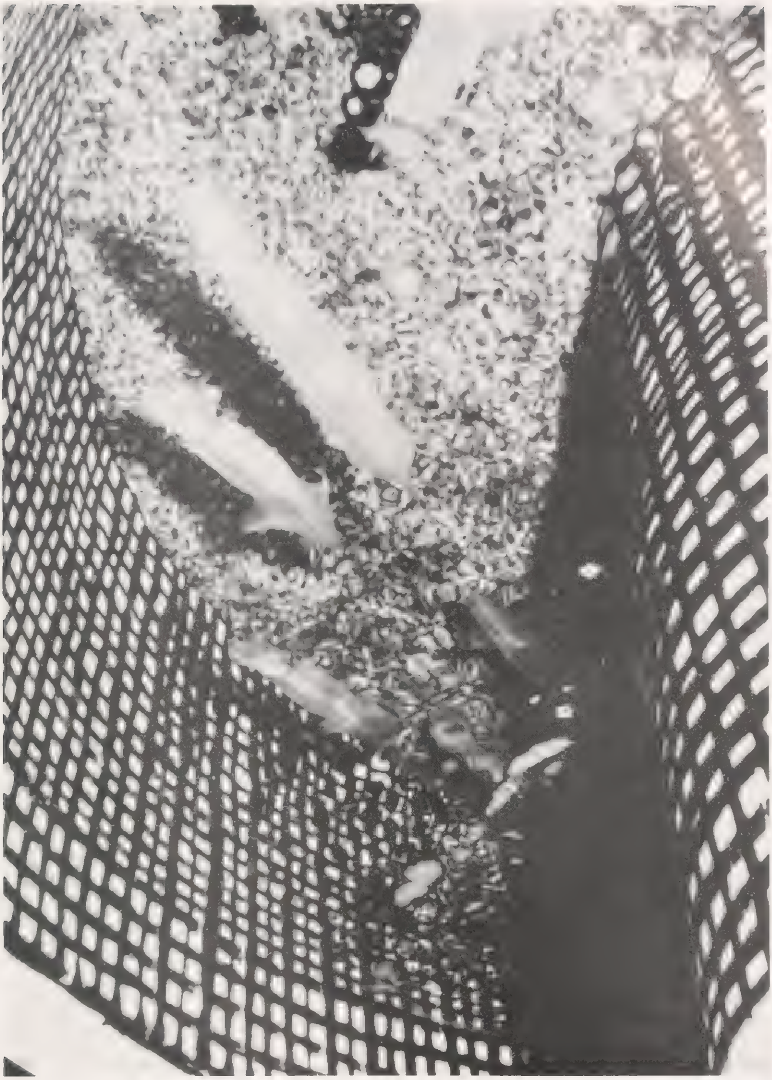


FIGURE 2. Tank walls' painted net patterns helped squid avoid collisions with the walls.

improve survival. When the tickness of zeolite layer in filter 1 was reduced to the original 5 cm, squid survival became similar to that of our previous experiment. Thus, improved survival was a function of zeolite. To further test the effect of zeolite upon survival, we tried three runs of 15, 12, and 5 squid, respectively, with filter 1 containing 80 kg zeolite. Average survival was 4.5 days (thin solid line in Fig. 3). Thus, 80 kg zeolite may be harmful to the squid. Probably, there exists an optimum amount of zeolite for survival. In our system, 20–40 kg of zeolite appeared optimum among amounts tested: 5, 20, 40, and 80 kg.

Maintenance of fed squid

Longer maintenance of squid (*Doryteuthis bleekeri*) by feeding them was tried February through April, 1979. Ten squid (one female, the rest male) were placed in the aquarium at the end of February. Four to five red goldfish (*Carassius auratus*) 4–5 cm in length per squid were put into the tank for food every day after the third day from the beginning of the experiment. Feeding was conducted twice a day, at 9 a.m. and 5 p.m. At first the fish were not eaten, but after about a week, squid ate the fish within 30 sec after the fish were put into the aquarium.

Figure 3 shows survival of 10 squid over a 2-month span. The survival times were 43–60 days. The first squid (female) died after spawning. Five squid that survived for over a month died shortly after their skins were scraped at the mantle tips. Once this area of skin was scraped, the squid rubbed their mantle tips more frequently on the tank walls. Another experience indicated that even a slight injury at the tip was fatal: When we stapled a small mark sheet on the squid to discriminate old from new squids, those stapled at their mantle tips died sooner.

DISCUSSION

Our previous report (Matsumoto, 1976) suggested that the cause of death of squid in a closed-system aquarium, even with enough oxygen dissolved in the sea water, was primarily the squids' lack of oxygen-adsorbing ability. Our present experiment further suggests that an obstacle to oxygen adsorption is organic substances generated in the aquarium or a substance secondarily produced from these organic substances, which can be overcome by using zeolite in a filter. In maintaining squid (*Illex illecebrosus*) for more than 30 days in an open-system tank of 15 m in diameter, O'Dor *et al.* (1977) reported that sea water was pumped from the Northwest Arm of Halifax Harbor through intakes located at a depth of 15 m, 0.7 m off the bottom, and that the water quality was relatively high.

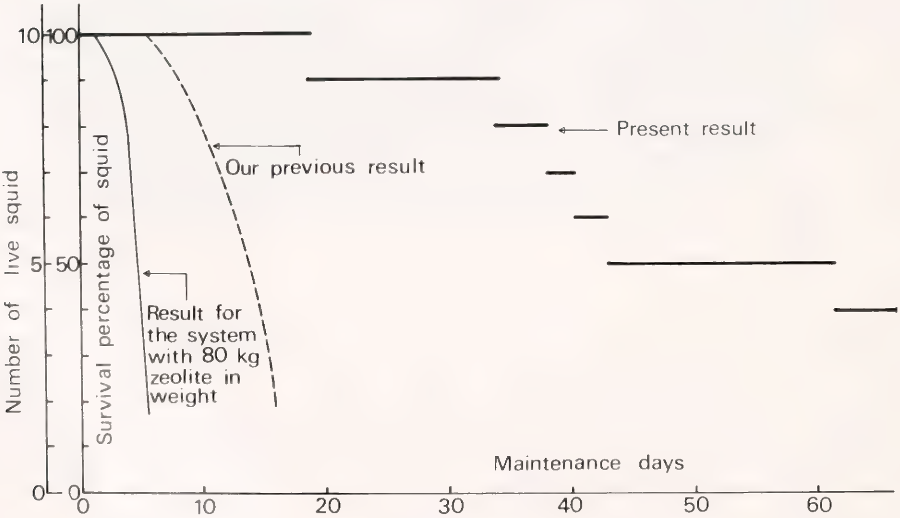


FIGURE 3. Survival of 10 squid over 2 months. The dashed line shows an old record of squid survival over 8 days (Matsumoto, 1976). The fine solid line shows results obtained for the system in Figure 1 when filter 1 contained 80 kg of zeolite. Calibration of squid survival record (discontinuous solid line) is in numbers of live squid, while calibrations for both fine solid and dashed lines (continuous lines) are in percentage of squid surviving.

With a closed or open system for maintenance of squid in the aquarium, high quality sea water seems to be crucial for squid survival.

In squid maintained for more than a month, skin damage was found to be fatal. In our maintenance system, the peripherally circulating flow of sea water, net patterns drawn on the tank walls, and continuous light enabled the squid to avoid hitting the walls. However, skin damage was caused by faint but repeated contacts with the tank walls, rather than by a harsh collision with the wall as suggested by Summers *et al.* (1974). Flores *et al.* reported that mortality after a 10 day survival test of squid (*Todarodes*) in an open system was high with the autumn-captured squid, possibly because of spread of a skin infection. O'Dor *et al.* (1977) concluded that deaths during the first week after specimens of *Illex illecebrosus* were put into the tank were associated with skin damage during capture. The reason why skin abrasion is fatal is not yet well understood. One explanation may be that skin abrasion provides an invasion site for bacteria, resulting in a disease leading to a quick death (Leibovitz *et al.*, 1977; Hulet *et al.*, 1979).

The condition of the squid when they were put into the tank obviously affected survival, as concluded by Summers *et al.* (1974) and O'Dor *et al.* (1977). In this respect, our squid-capturing method using Japanese squid jiggers (Matsumoto, 1976) is satisfactory. Initial condition also depends upon time between capture and arrival at a laboratory. In our case, shipboard transportation required 3–10 hr, and trucking from the seashore to our laboratories at Tanashi and Tsukuba took 3–5 and 6–12 hr, respectively. When truck transport took more than 8 hr, some squid were found to lie down on the bottom of the transportation tank. They usually recovered in the aquarium, but died within a week.

Two-week survival tests showed the importance of feeding squid. We tried live red goldfish and frozen sardines as food, and found the goldfish more suitable. Small frozen sardines, 9–12 cm long, were purchased from a fish seller. One sardine per squid a day was defrosted, tied at the tail by white thread and hung in the aquarium until captured by squid. The fatty sardines made the sea water oily, so that we had to make the sea water overflow by adding reserved sea water to the aquarium. Goldfish (*Carassius auratus*) usually live in fresh water, and do not survive in sea water longer than 10 min. However, squid were able to catch the live goldfish within 30 sec after the fish were put in the aquarium. The live goldfish were cheap (about 4 cents each), and squid can swallow them, so that sea water is kept clean.

Efforts to provide a steady supply of squid for neurosciences should now be directed to rearing from eggs in the aquarium (LaRoe, 1971; von Boltzky, 1971).

SUMMARY

Ten adult squid (*Doryteuthis bleekeri*) were put into a small (1.37 m^2 in area) circular tank in a closed system. Half of the squid survived 43–60 days, the other half longer. Filtering ability was essential to squid maintenance, and filtering ability was satisfactorily supported by zeolite in appropriate amounts. In long-term (over a month) maintenance, skin damage caused by faint but repeated contacts with the tank walls became a major cause of death. Feeding squid was found to be important for squid maintenance, and live goldfish (*Carassius auratus*) were found to be satisfactory as food for long-term maintenance of squid.

LITERATURE CITED

Arnold, J. M., W. C. Summers, D. L. Gilbert, R. S. Manalis, N. W. Daw, and R. J. Lasek, 1974. *A guide to the laboratory use of the squid* Loligo pealei. Marine Biological Laboratory, Woods Hole, Massachusetts. Pp. 9–17.

Flores, E. E. C., S. Igarashi, and T. Mikami, 1977. Studies on squid behavior in relation to fishing. II. On the survival of squid, *Torarodes pacificus Steenstrup*, in experimental aquarium. Bull. Fac. Fish. Hokkaido Univ., **28**: 137–142.

Hanlon, R. T., R. F. Hixon, and W. H. Hulet, 1978. Laboratory maintenance of wild-caught loliginid squids. *Fisheries and Marine Service Technical Report*, **833**: 20. 1–20.14.

Hulet, W. H., M. R. Villoch, R. F. Hixon, and R. T. Hanlon, 1979. Fin damage in captured and reared squids. Lab. Anim. Sci. **29**: 528–533.

Leibovitz, L., T. R. Meyers, R. Elston, and P. Chaney, 1977. Necrotic exfoliative dermatitis of captive squid (*Loligo pealei*). *J. Invertebr. Pathol.*, **30**: 369–376.

LaRoe, E. T., 1971. The culture and maintenance of the loliginid squids *Sepioteuthis sepioidea* and *Doryteuthis plei*. *Mar. Biol.*, **9**: 9–25.

Matsumoto, G. 1976. Transportation and maintenance of adult squid (*Doryteuthis bleekeri*) for physiological studies. *Biol. Bull.*, **150**: 279–285.

O'Dor, R. K., R. D. Durward, and N. Balch, 1977. Maintenance and maturation of squid. (*Illex illecebrosus*) in a 15 meter circular pool. *Biol. Bull.*, **153**: 322–335.

Summers, W. C., and J. J. McMahon, 1974. Studies on the maintenance of adult squid (*Loligo pealei*). I. Factorial survey. *Biol. Bull.*, **146**: 279–290.

Summers, W. C., J. J. McMahon, and G. N. P. A. Ruppert, 1974. Studies on the maintenance of adult squid (*Loligo pealei*). II. Empirical extensions. *Biol. Bull.*, **146**: 291–301.

Von Boletzky, S., M. V. Von Boletzky, D. Frosch, and V. Gotzi. 1971. Laboratory rearing of Sepiolinae (Mollusca: Cephalopoda). *Mar. Biol.*, **8**: 82–87.

Reference: *Biol. Bull.*, **159**: 325–336. (October, 1980)

SODIUM TRANSPORT IN THE FRESHWATER ASIATIC CLAM *CORBICULA FLUMINEA*

SUSAN McCORKLE AND THOMAS H. DIETZ

Department of Zoology and Physiology, Louisiana State University, Baton Rouge, LA 70803

Freshwater bivalves have a low blood-solute concentration that reduces the concentration gradients between their body fluids and the external medium and minimizes passive ion movements (Dietz and Branton, 1975; Potts, 1954; Prosser, 1973). Ion loss is still a problem due to the hyperosmotic blood and must be balanced by active ion uptake. Active Na and Cl transport systems have been reported in freshwater bivalves (Dietz, 1978, 1979; Krogh, 1939), the possible sites of Na transport being the mantle, kidney, and gill epithelia (Saintsing and Towle, 1978).

Members of the molluscan superfamily Sphaerioidea have a higher rate of Na transport than do members of the superfamily Unionoidea. The sphaeriid species *Corbicula fluminea* (=*C. manilensis*) and *Musculium transversum* (=*Sphaerium transversum*) transport Na at rates up to twice those of the Unionoidea, approaching the rates of brackish water species (Dietz, 1979). *Corbicula fluminea* is considered to be a freshwater animal although it has been reported to inhabit brackish water up to 5‰ salinity (Filice, 1958; Hayashi, 1956). Geologically, the Corbiculidae are recent invaders of freshwater (Keen and Casey, 1969).

Few physiological studies have been reported for *C. fluminea* (Dietz, 1979; Gainey, 1978a, b; Gainey and Greenberg, 1977). Because this species has been reported in both estuarine and freshwater habitats, the present study was undertaken to elucidate its mechanism of Na regulation. Sodium balance in *C. fluminea* was examined by partitioning Na flux into four processes: 1) active transport, in which ions are moved against electrochemical gradients, 2) passive diffusion, 3) exchange diffusion, an obligatory exchange of an ion with an identical ion located on the opposite side of a membrane, and 4) excretion.

MATERIALS AND METHODS

Specimens of *Corbicula fluminea* were collected from local bayous and maintained unfed in an aerated aquarium containing artificial pondwater (0.5 mM NaCl, 0.4 mM $CaCl_2$, 0.2 mM $NaHCO_3$, 0.05 mM KCl). The clams were placed in a 12L:12D photoperiod at room temperature (20°–25°C) and were allowed to acclimate to laboratory conditions for at least 7 days before use. Animals undergoing salt depletion were placed in deionized water that was changed frequently. Salt depletion for more than 3 months resulted in less than 5% mortality.

Measurement of sodium flux

Sodium influx was determined from calculations based on the slope of a line representing a decrease in ^{22}Na radioactivity of the bathing medium as a function of time (Dietz, 1978, 1979; Dietz and Branton, 1975).

All experiments were begun at approximately 11 a.m. to minimize the influence of physiological rhythms (McCorkle *et al.*, 1979). Clams were placed in a deionized water bath for 1 hr prior to each study to remove adsorbed ions and waste products from the valves and mantle cavity. Clams were then moved to individual 50-ml beakers containing 30–40 ml of a bathing solution. The volume of the bath depended upon the type of experiment. Except where noted, the bathing solution was Na_2SO_4 to eliminate any Na Cl transport interactions, as the SO_4 ion is non-permeating (Dietz, 1978; Ehrenfeld, 1974). Within an hour the valves opened and the animals were siphoning the bath. Turbulence at the air-bath interface verified that the animals were in contact with and circulating the bathing medium. An initial bath sample was taken at 0 hr with a second sample taken 1–3 hr after the onset of the flux study. Sample volumes were not replaced. The time interval between samples allowed a 10–20% decrease in bath radioactivity. Bath volume and time interval were varied to prevent more than a 25% decrease in the radioactivity of the bathing medium. After the second sample was taken, the animals were removed from the bath, drained of mantle-cavity water, blotted dry, and weighed for determination of total wet weight. The valves were opened and the soft tissues removed and oven-dried at 95°C for 24 hr for measurement of dry tissue weight, shell excluded.

Sodium concentrations were measured using a flame photometer. A Triton X-100, toluene, p-terphenyl scintillation "cocktail" was added to an aliquot of each bath sample, and radioactivity was assayed by a liquid scintillation counter.

Net Na flux (J_n^{Na}) was calculated from changes in the Na concentration of the bathing medium. The net flux is positive when there is a net absorption of the ion by the animal, and negative when there is a net loss of the ion to the external medium. Unidirectional Na influx (J_i^{Na}), ions taken up from the bath by the animal, was calculated from the rate of ^{22}Na disappearance from the bath. Sodium efflux (J_o^{Na}), ions lost by the animal to the bath, was determined from the relationship $J_o = J_i - J_n$. All flux rates are given as μM Na/(g dry tissue ·hr).

Blood ion composition

Blood samples were obtained from the clams by cardiac puncture (Fyhn and Costlow, 1975). The blood was centrifuged for 2 min at 8000 × *g* to remove the cells, and the supernatant used for ion analyses. Chloride concentrations were measured by electrometric titration. Total solutes were determined from undiluted blood using a freezing-point depression osmometer.

Identification of exchange ions

The pH of the bathing medium was measured at 0 and 3 hr during Na flux study to estimate the net H efflux (J_n^H). The bath was buffered initially to pH 7.3 with 1 mM tris (hydroxymethyl) aminomethane to stabilize the pH. Bath samples were sonicated for 2 min to remove dissolved respiratory CO_2. A pH decrease was noted in each experiment. The 3-hr sample was titrated back to the original pH at 0 hr with 4.8 mN NaOH to determine J_n^H.

The release of NH_4 by the clams was assayed using the phenolhypochlorite method of ammonia determination (Solorzano, 1969). Five-ml bath samples were taken at 0 and 3 hr during an Na flux experiment. Due to tris interference in NH_4 determinations, an unbuffered 0.5 mM Na_2SO_4 medium was used. Net NH_4 flux ($J_n^{NH_4}$) was calculated from the difference between the 3- and 0-hr NH_4 concentrations.

Amiloride (0.5 and 1.0 mM/l) and ouabain (0.5 mM/l), both potential inhibitors of Na transport, were tested in the Na_2SO_4 bath as aids to identification of the possible exchange ion(s) for Na. Amiloride is thought to act on the outside surface of the epithelium, reversibly blocking the entry of Na (Ussing *et al.*, 1974). Inhibition of Na influx coupled with inhibition of H efflux has been demonstrated in crayfish, rainbow trout, frogs, and the unionid *Carunculina texasensis* (Ehrenfeld, 1974; Kirschner *et al.*, 1973; Dietz, 1978). Ouabain specifically inhibits the activity of Na-K ATPases involved in Na transport. Inhibition of NH_4 efflux by ouabain occurred in the estuarine clam, *Rangia cuneata* (Mangum *et al.*, 1978), possibly indicating an inhibitory effect of ouabain on Na/NH_4 exchange.

Partitioning of the sodium flux

Active Na transport can be measured with a radioactive tracer. To obtain a complete picture of ion balance it is necessary to quantify passive ion movements by diffusion, excretion, and exchange diffusion. Not all Na movement is active transport.

The influx (J_i) and efflux (J_o) values recorded in Tables II and III and in Figures 1 and 2 are composites of active transport, diffusion, exchange diffusion, and excretion (for efflux only), representing the total unidirectional movement of Na by *C. fluminea*. Separate studies were performed to partition the individual components of Na movement. The components of total influx (J_i^{Total}) and total efflux (J_o^{Total}) are:

$$J_i^{Total} = \text{Exchange Diffusion} + \text{Passive Inward Diffusion} + \text{Active Transport}$$
$$J_o^{Total} = \text{Exchange Diffusion} + \text{Passive Outward Diffusion} + \text{Excretion.}$$

The J_o^{Total} was calculated from the relationship: $J_o = J_i - J_n$. The diffusive and excretory Na losses cannot be separated (Dietz and Branton, 1979) and were measured together as the net loss of Na (J_n^{Na}) to deionized water. The animals were placed in individual beakers containing deionized water for 3 hr and the appearance of Na in the bath was measured. The animals were returned to pondwater for 2 days and then used in a unidirectional flux study in 0.5 mM Na_2SO_4. After an additional 2 days in pondwater J_n^{Na} was again measured in deionized water. The average value for J_n^{Na} in deionized water was defined as the combined diffusive/excretory Na component of J_o^{Total}. Since there was initially no Na in the bath to be actively exchanged for internal H or NH_4 or to participate in exchange diffusion, any Na in the deionized water at the end of 3 hr was assumed to have been lost from the clams by passive outward diffusion and/or excretion. The diffusive/excretory Na loss in Na_2SO_4 is assumed to be unchanged from the loss in deionized water. By subtracting the diffusive/excretory Na loss from J_o^{Total}, an estimated value for exchange diffusion was obtained.

The J_i^{Total} was calculated from changes in bath radioactivity and changes in the Na concentration of the bathing medium over time. By definition, the exchange diffusion out of the animal is equal to exchange diffusion into the animal. The exchange diffusion value obtained for Na efflux was inserted into the equation for J_i^{Total} as the amount of Na influx due to exchange diffusion. Transepithelial potentials (TEP) were measured *in vivo* (see below) in order to calculate the passive inward diffusion component of J_i^{Total}. Maximum passive inward diffusion of Na was calculated from Ussing's flux ratio equation (Ussing, 1949):

$$J_i^{Diffusion} = (J_o^{Diffusion+Excretion} \cdot C_o/C_i) \exp FE/RT$$

where J_o is assumed to be entirely diffusion, and C_o and C_i are the Na concentrations of the bathing medium and blood of *C. fluminea* (Table I), respectively. Faraday's constant is represented by F, E is the TEP, R is the gas constant in electrical units, and T is absolute temperature. The value for active transport was estimated by subtracting the exchange diffusion and passive inward diffusion components from J_i^{Total}.

Transepithelial potentials (TEP)

To measure the TEP, a small hole was drilled in the umbonal region of the dorsal valve approximately 4 mm ventral to the hinge line. A 2 cm piece of glass tubing was epoxied over the hole. Polyethylene tubes filled with 20 mM KCl-3% agar were connected to calomel electrodes which were, in turn, connected to a recording potentiometer. One KCl bridge was inserted through the glass tube into the body fluids. A second bridge was placed in the 0.5 mM Na_2SO_4 bathing medium as a reference. Potentials were measured when the valves were open and the clams were siphoning the bathing medium.

Statistical analyses

Student's *t* test was used to compare means of different groups of clams. The significance of the correlation between J_i^{Na} and the sum of J_n^{H} and $J_n^{NH_4}$ was tested using linear regression. Test statistics beyond the 95% region of acceptance were considered significant ($P < 0.05$) and statistics falling outside of the 99% region of acceptance were considered highly significant ($P < 0.01$). All data appear as mean ± 1 standard error.

RESULTS

Effects of pondwater acclimation vs. salt depletion

A comparison of the dry tissue weights and percent body water of salt-depleted clams and clams acclimated to pondwater showed significant differences between the two groups (Table I). There was no significant difference in dry shell weights, but there was a significant difference in total wet weights. The total weight of salt-depleted clams was significantly higher than the total weight of clams acclimated to pondwater. Salt-depleted animals had a smaller dry tissue weight, but the percent body water was significantly higher than that of animals acclimated to pondwater.

TABLE I

Comparison of tissue weights, percent body water and blood ion composition of salt-depleted and pondwater-acclimated Corbicula fluminea. *Data are presented as mean ± SEM* (* $P < 0.05$, ** $P < 0.01$).

	Total wet weight (g)	Dry shell (g)	Dry tissue (mg)	% body water	Total blood solutes (mOsm)	mM/l	
						Na	Cl
Pondwater	5.074 ± 0.072	2.954 ± 0.049 (N = 64)	98 ± 3	95.4 ± 0.1	54.6 ± 0.6	23.5 ± 0.3 (N = 10)	24.2 ± 0.7
Salt-depleted	5.236 ± 0.113*	3.049 ± 0.070 (N = 45)	77 ± 2**	96.4 ± 0.1**	42.1 ± 0.5**	19.0 ± 0.2** (N = 10)	17.3 ± 0.4**

TABLE II

Sodium flux of salt-depleted and pondwater-acclimated Corbicula fluminea *in a 0.5 mM Na_2SO_4 bathing medium. Data appear as mean ± SEM* (** $P < 0.01$).

Treatment	N	μM Na/(g dry tissue·hr)		
		J_n	J_i	J_o
Pondwater	32	−2.67 ± 0.86	7.90 ± 0.79	10.57 ± 0.95
Salt-depleted	35	9.79 ± 1.23**	18.53 ± 2.10**	8.74 ± 1.27

Salt depletion caused a marked reduction in Na and Cl ions in the blood of *C. fluminea* (Table I). The total blood-solute level was reduced by 23% in salt-depleted clams, and blood Na and Cl concentrations dropped significantly. The combined decrease of Na and Cl in the blood accounted for 91% of the decrease in total blood solutes of salt-depleted clams.

Sodium flux

Salt depletion enhanced the rate of unidirectional Na uptake from an Na_2SO_4 bathing medium (Table II). The J_i^{Na} increased 234% in salt-depleted clams compared to pondwater-acclimated animals ($P < 0.01$). The J_o^{Na} was not significantly different in pondwater and salt-depleted clams. Due to increased J_i^{Na} in the salt-depleted animals, J_n^{Na} was positive.

Kinetics of sodium transport

The rate of Na influx was dependent on the external Na concentration in pondwater-acclimated and salt-depleted *C. fluminea*. A non-linear relationship was found between J_i^{Na} and external Na concentration (Fig. 1). The transport system was saturable. The maximum rate of J_i^{Na}, $\dot{V}_{max}$, for pondwater clams was 12.90 ± 3.01 μM Na/(g dry tissue·hr) in a 0.87 mM Na/l bathing solution. The affinity of the transport system, K_m, is the external Na concentration at which half of the maximum rate of Na influx occurs. The affinity of pondwater clams was 0.05 mM Na/l. Sodium influx of salt-depleted clams was variable, but the influx rates were always higher than the rates of pondwater animals in

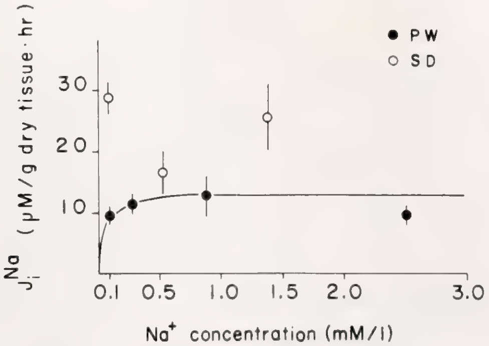


FIGURE 1. Effect of Na_2SO_4 concentration of the bathing medium on unidirectional sodium influx in *Corbicula fluminea*. Each point represents mean ± SEM for five animals either acclimated to pondwater (PW) or salt-depleted (SD).

TABLE III

The effects of amiloride and ouabain on sodium transport in pondwater-acclimated specimens of Corbicula fluminea. *Amiloride and ouabain were dissolved in 0.5 mM Na_2SO_4. Data are presented as mean ± SEM* (* $P < 0.05$).

Treatment	N	μM Na/(g dry tissue·hr)		
		J_n	J_i	J_o
Control	16	−0.04 ± 1.49	7.26 ± 1.01	7.29 ± 1.40
Amiloride, 0.5 mM and 1.0 mM	11	4.71 ± 1.66*	9.26 ± 1.83	4.55 ± 1.23
Ouabain, 0.5 mM	11	−3.28 ± 2.62	7.09 ± 1.13	10.37 ± 2.49

all bath concentrations tested. The maximum transport rate for salt-depleted animals was 28.66 ± 2.17 μM Na/(g dry tissue·hr). From the $\dot{V}_{max}$, K_m was estimated for salt-depleted clams as 0.04 mM Na/l.

Effects of amiloride and ouabain on sodium transport

Amiloride at concentrations of 0.5 and 1.0 mM/l resulted in no significant changes in J_i^{Na} or J_o^{Na} and the data were pooled (Table III). Although there appeared to be a significant difference between control and amiloride-treated net fluxes, the difference may be an artifact. The fluxes from individual experiments using amiloride were not consistent. The treatment appeared to be stimulatory, inhibitory, or showed no effect on rates of Na flux at both concentrations. Ouabain (0.5 mM/l) added to the bathing solution had no effect on Na fluxes.

Partitioning of the sodium flux

The outward movement of Na to deionized water ($J_o^{Diffusion} + J_o^{Excretion}$) was 2.87 ± 0.76 μM Na/(g dry tissue·hr) for pondwater-acclimated clams (Table IV). Rates of diffusive and excretory Na loss measured in Na_2SO_4 were assumed to be unchanged from the rates measured in deionized water. The J_o^{Total} for pondwater clams was 8.94 ± 0.76 μM Na/(g dry tissue·hr). Therefore, 5.91 ± 0.80 μM Na/(g dry tissue·hr) was the estimated value for exchange diffusion. The outward diffusive/excretory component of Na movement decreased significantly ($P < 0.05$) from 2.87 ± 0.76 μM Na/(g dry tissue·hr) to 0.87 ± 0.38 μM Na/(g dry tissue·hr) in salt-depleted animals.

Total inward movement of Na, J_i^{Total}, was 8.82±0.60 μM Na/(g dry tissue·hr) for pondwater clams (Table V). The maximum passive diffusive influx was estimated with Ussing's flux ratio equation and was 0.50 μM Na/(g dry tissue·hr) for pondwater-acclimated clams (Table V). The inward diffusion of Na doubled in salt-depleted clams. The measured potential difference across the epithelia was −7.0 ± 1.2 mV (N = 10), blood negative to the bathing medium. There was no significant difference between the TEPs of pondwater-acclimated and salt-depleted specimens of *C. fluminea* and the data were pooled. The active transport component of Na movement was estimated to be 2.41 μM Na/(g dry tissue·hr) for pondwater-acclimated clams and increased fivefold in salt-depleted animals. Salt-depleted clams exhibited highly significant increases in rates of exchange diffusion, total Na influx, and total Na efflux (Tables IV and V).

TABLE IV

A partitioning of unidirectional sodium efflux in Corbicula fluminea. *Total sodium efflux is the unidirectional efflux measured in a 0.5 mM* Na_2SO_4 *bath. The diffusive/excretory component was measured in a deionized water bath. Exchange diffusion was calculated as* $J_o^{\text{Total}} - (J_o^{\text{Diffusion}} + J_o^{\text{Excretion}})$. *Data appear as mean ± SEM* (* $P < 0.05$, ** $P < 0.01$). † *Means were calculated from individual animals and this accounts for the lack of agreement with the average value*

Treatment	μM Na/(g dry tissue·hr)				
	$J_o^{\text{Total Na}}$	=	$J_{o\,\text{Diffusion}}^{\text{Exchange}}$	+	$(J_o^{\text{Diffusion}} + J_o^{\text{Excretion}})$
Pondwater	8.94 ± 0.76 (N = 6)		5.91 ± 0.80† (N = 6)		2.87 ± 0.76 (N = 11)
Salt-depleted	16.92 ± 0.84** (N = 7)		16.05 ± 0.67** (N = 7)		0.87 ± 0.38* (N = 14)

Exchange ions

Hydrogen and ammonium ions may be exchanged for Na in *C. fluminea*. Net ammonium flux was measured in a 0.5 mM Na_2SO_4 bath and a deionized water bath to determine the NH_4 loss through active transport and the diffusive/excretory NH_4 loss. The value of $J_n^{NH_4}$ in deionized water was not significantly different from the $J_n^{NH_4}$ in Na_2SO_4, indicating that NH_4 efflux in pondwater-acclimated clams was mostly passive and not involved in exchange for Na ions (Table VI). The $J_n^{NH_4}$ of salt-depleted animals in deionized water, 1.28 ± 0.18 μM NH_4/(g dry tissue·hr), was not significantly different from the $J_n^{NH_4}$ of pondwater animals. However, the $J_n^{NH_4}$ when salt-depleted animals were in a Na_2SO_4 bathing medium increased to 4.15 ± 0.18 μM NH_4/(g dry tissue·hr), a highly significant rise in NH_4 efflux.

Net hydrogen ion flux, J_n^H, in pondwater animals was 5.12 ± 0.53 μM H/(g dry tissue·hr) (Table VI). The J_n^H of salt-depleted animals, 7.62 ± 2.00 μM H/(g dry tissue·hr), was not significantly different from the J_n^H of pondwater animals. The sum of J_n^H and $J_n^{NH_4}$ in Na_2SO_4, 11.77 μM/(g dry tissue·hr), balanced 83% of the Na actively transported into the salt-depleted animals (Fig. 2). A linear regression of J_i^{Na} and $J_n^{H+NH_4}$ for pondwater and salt-

TABLE V

A partitioning of the unidirectional influx of sodium in Corbicula fluminea. *Total sodium influx is the unidirectional influx measured in a 0.5 mM* Na_2SO_4 *bath. Inward diffusion of sodium was calculated from the flux ratio equation. Active transport was calculated as* $J_i^{\text{Total}} - (J_{i\,\text{Diffusion}}^{\text{Exchange}} + J_i^{\text{Diffusion}})$. *Data appear as mean ± SEM* (** $P < 0.01$).

Treatment	μM Na/(g dry tissue·hr)						
	$J_i^{\text{Total Na}}$	=	$J_{i\,\text{Diffusion}}^{\text{Exchange}}$	+	$J_i^{\text{Diffusion}}$	+	$J_{i\,\text{Transport}}^{\text{Active}}$
Pondwater	8.82 ± 0.60 (N = 6)		5.91 ± 0.80 (N = 6)		0.50		2.41
Salt-depleted	31.42 ± 1.39** (N = 7)		16.05 ± 0.67** (N = 7)		1.17		14.20

TABLE VI

A comparison of the net NH_4 flux in 0.5 mM Na_2SO_4 and deionized water baths and net H flux in 0.5 mM Na_2SO_4 of pondwater-acclimated and salt-depleted Corbicula fluminea. *Data appear as mean ± SEM* (** $P < 0.01$). *Means that are not significantly different are indicated by N.S.*

Treatment	μM/(g dry tissue·hr)		
	$J_n^{NH_4}$ in Na_2SO_4	$J_n^{NH_4}$ in deionized H_2O	J_n^H in Na_2SO_4
Pondwater	1.17 ± 0.22 (N = 6)	1.77 ± 0.18 (N = 14)	5.12 ± 0.53 (N = 10)
	N.S.		
Salt-depleted	4.15 ± 0.18** (N = 7)	1.28 ± 0.18 (N = 14)	7.62 ± 2.00 (N = 7)
	**		

depleted animals resulted in the significant regression coefficient value $r = 0.66$ ($P < 0.02$).

DISCUSSION

Corbicula fluminea exhibits an Na transport mechanism different from that of the unionid mussels. The high J_i^{Na} and J_o^{Na} for *C. fluminea* agree with those pre-

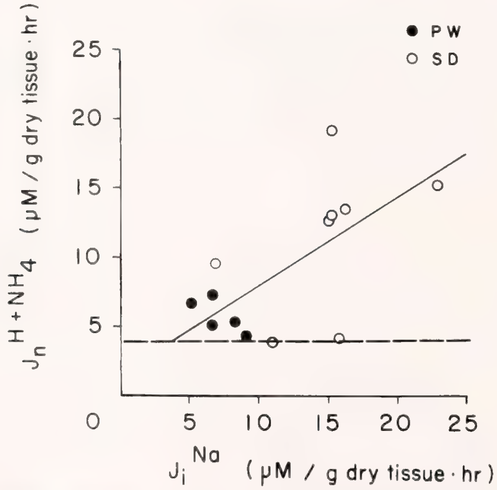


FIGURE 2. Relationship between unidirectional sodium influx and the sum of the net loss of H and NH_4 of pondwater-acclimated (PW) and salt-depleted (SD) *Corbicula fluminea* in 0.5 mM Na_2SO_4. The broken line represents the baseline excretory H + NH_4 efflux for pondwater-acclimated clams. The equation for the regression line is $J_n^{H+NH_4} = (1.48 \pm 0.80) + (0.65 \pm 0.60)J_i^{Na}$. The regression coefficient, r, is equal to 0.66 ($P < 0.02$).

viously reported by Dietz (1979). Sodium influx of *C. fluminea* is approximately six times the rate of the unionids *Carunculina texasensis* and *Ligumia subrostrata* and twice that of *Margaritifera hembeli* (Dietz, 1978, 1979). Sodium efflux is an order of magnitude higher than that of any of the unionid mussels studied by Dietz (1979). *Corbicula fluminea* also has a higher Na uptake rate relative to other freshwater invertebrates examined: 1.28 μM Na/(g wet tissue·hr) as compared to 0.29 μM Na/(g wet tissue·hr) in the gastropod *Limnaea stagnalis*, 0.65 μM Na/(g wet tissue·hr) in the crayfish *Astacus pallipes* and 0.70 μM Na/(g wet tissue·hr) (at 18°C) for the earthworm *Lumbricus terrestris* (Greenaway, 1970; Shaw, 1959; Dietz and Alvarado, 1970).

The major difference between the fluxes of *C. fluminea* and other freshwater animals is the presence of a substantial exchange diffusion component. Sixty-seven percent of the total inward Na movement is due to exchange diffusion. Only 22% of the J_i^{Na} in the crayfish and minimal amounts of the J_i^{Na} in *Carunculina texasensis* are attributable to exchange diffusion (Shaw, 1959; Dietz, 1978). In addition to the large percentage of exchange diffusion, the rate of active transport of Na into pondwater-acclimated specimens of *C. fluminea*, 2.41 μM Na/(g dry tissue·hr), is almost double the approximate value of 1.3 μM Na/(g dry tissue·hr) recorded for the unionids (Dietz, 1979). Absence of the active transport component could place the animals in a negative ion balance, depending on the rate of Na efflux relative to the inward Na movement.

The magnitude of active Na transport depends upon the degree of salt depletion. Table II indicates transport rates for animals salt-depleted for an average of 40 days. Although the fluxes were not partitioned for these studies, the active transport component is at least equal to the net Na uptake. The clams used for the partitioning studies were salt-depleted for a longer period (about 90 days) and display a further elevation of active Na transport (Table V). For all studies, Na-Na exchange diffusion is about half of the total Na influx. Exchange diffusion is characteristic of corbiculid Na transport.

Non-equilibrium conditions may be inferred by comparing observed TEP with TEP estimated for animals in a steady-state condition. In a steady state Ussing's flux ratio equation simplifies to the Nernst equation:

$$E = (RT/F) \ln (C_i/C_o),$$

giving the TEP (E) necessary to maintain Na in electrochemical equilibrium. If there is no active transport, observed TEP would equal the TEP estimated by the Nernst equation. The estimated TEP of −74 mV does not equal the observed TEP of −7 mV, suggesting active Na transport in *C. fluminea*. Previous studies on other freshwater clams have indicated that TEP is a calcium-diffusion potential independent of Na, Cl, or SO_4 (Dietz and Branton, 1975).

Transport of Na in *C. fluminea* is efficient. The affinity of the transport system for Na ions in these animals is greater than the affinity in the unionid mussels. Even with twice the active transport rate the affinity, K_m, in *C. fluminea* is 0.05 mM Na/l as compared to the K_m of 0.15 mM Na/l in *Carunculina texasensis* (Dietz, 1978). The K_m in *C. fluminea* is low relative to literature K_m values of 0.2–0.7 mM Na/l in other freshwater animals (Dietz and Alvarado, 1970, 1974; Greenaway, 1970; Maetz, 1973; Prosser, 1973; Shaw, 1959). Sodium influx increases two to three times in salt-depleted animals without a significant change in K_m, suggesting that more epithelial transport sites are activated during the salt-depletion treatment. Passive diffusion and/or excretion of Na out of the salt-depleted animal is reduced (Table IV).

The active transport systems in *Carunculina texasensis* and several other freshwater animals are primarily Na/H exchanges with minimal involvement of NH_4 as an exchange ion (Dietz, 1978, 1979; Ehrenfeld, 1974; Kerstetter *et al.*, 1970; Maetz, 1973; Maetz *et al.*, 1976). *Corbicula fluminea* acclimated to pondwater does not differ from other freshwater animals in this respect. The $J_n^{NH_4}$ of pondwater-acclimated clams appears to be primarily excretory since no change in NH_4 output was noted between Na_2SO_4 and deionized-water bathing solutions. Although the J_n^H was not measured in a deionized water bath, the excretory J_o^H may be estimated. Assuming that Na/H exchange occurs as a 1:1 ratio, active Na transport may be subtracted from total J_n^H, leaving 2.71 μM H/(g dry tissue·hr) as the excretory output. Na/H exchange has been reported to occur on a 1:1 basis in two species of amphibia and in crayfish (Garcia Romeu *et al.*, 1969; Garcia Romeu and Ehrenfeld, 1975; Ehrenfeld, 1974). The baseline excretory $H + NH_4$ efflux for pondwater animals may then be approximated as 4.12 μM/(g dry tissue·hr) (Fig. 2).

Salt-depleted and pondwater-acclimated specimens of *C. fluminea* show essentially the same $J_n^{NH_4}$ in deionized water; however, the $J_n^{NH_4}$ for salt-depleted clams in Na_2SO_4 quadrupled. Although Na/H exchange appears to be the primary exchange mechanism under normal conditions (Maetz and Garcia Romeu, 1964; Maetz *et al.*, 1976), *C. fluminea* may be activating a Na/NH_4 exchange as an auxiliary mechanism under stress of salt depletion. The active transport exchange ratio of $Na : (H + NH_4)$ was 2.2:1 in salt-depleted clams. The lack of stoichiometry suggests there may be a change in epithelial Na permeability when animals are in Na_2SO_4 solutions. The calculated exchange diffusion in Na_2SO_4 may therefore by underestimated and active Na transport overestimated.

Salt depletion is often used to stimulate active Na uptake in freshwater animals, but important changes in the animals should be recognized. Salt depletion caused a significant reduction in dry tissue weight of *C. fluminea*. Concurrently, the percent body water increased. The total blood-solute level was reduced, mostly by highly significant reductions in Na and Cl concentrations. These animals have undergone salt-depletion in the laboratory for long periods of time without mortality, but part of the elevated Na influx may be due to the 20% loss in dry tissue weight. The small tissue weight of this particular clam species makes the changes in weight and body water even more important. Significant loss of dry tissue weight is correlated with elevated NH_4 efflux in salt-depleted clams. Tissue protein hydrolysis generates free amino acids, which may become major intracellular solutes contributing to water gain. Free amino acids are probably a major energy source under these laboratory conditions. Catabolism of amino acids would generate the additional H and NH_4 necessary for Na exchange in the salt-depleted clams.

Specimens of *Corbicula fluminea* show little sensitivity to amiloride and ouabain as inhibitors of Na transport. The apparent lack of inhibition in pondwater-acclimated animals may be a result of the small fraction of total Na turnover attributable to active Na transport. These data suggest a fundamental difference in the mechanism of Na–Na exchange diffusion and active Na transport, rather than exchange diffusion being an artifact of active transport.

The auxiliary Na/NH_4 exchange in salt-depleted animals may have been an important exchange mechanism when *C. fluminea* inhabited brackish water. Ammonia efflux against a gradient has been reported in the marine invertebrates *Rangia cuneata*, *Nereis succinea*, and *Callinectes sapidus* (Mangum *et al.*, 1976, 1978). The large exchange-diffusion component of *C. fluminea* might also be a vestige of its brackish water habitation. Exchange diffusion in estuarine blue crabs living in freshwater is reported to comprise 50% of Na influx (Cameron, 1978). Exchange diffusion in the unionids, long term inhabitants of freshwater (Haas, 1969), is negligible. Many of the differences in the Na balance mechanism of *Corbicula fluminea* as compared to other freshwater animals may be attributable to its recent brackish water heritage.

We thank Deborah M. McMullan for typing the manuscript. This paper was submitted by Susan McCorkle to L.S.U. in partial fulfillment of the requirements for the M.S. degree. This research was supported by NSF grants PCM75-05483 AO1 and PCM77-08818.

SUMMARY

The Na transport mechanism was examined in pondwater-acclimated (PW) and salt-depleted (SD) specimens of *Corbicula fluminea*. The Na influx in 0.5 mM Na_2SO_4 of 7.90 ± 0.79 μM Na/(g dry tissue·hr), higher than most freshwater animals, increased to 18.53 ± 2.10 μM Na/(g dry tissue·hr) in SD animals.

Saturation of the transport system is typical of Michaelis-Menten enzyme kinetics. Maximum influx of PW clams was 12.90 ± 3.01 μM Na/(g dry tissue·hr), with a K_m of 0.05 mM Na/l. The maximum rate in SD clams was 28.66 ± 2.17 μM Na/(g dry tissue·hr), with little change in K_m.

Sodium movement in *C. fluminea* may be partitioned into passive diffusion, excretion, exchange diffusion and active transport. Exchange diffusion comprises a substantial portion of Na movement: 5.91 ± 0.80 μM Na/(g dry tissue·hr) in PW animals and 16.05 ± 0.67 μM Na/(g dry tissue·hr) in SD clams. Passive inward diffusion of Na was 0.50 μM Na/(g dry tissue·hr) for PW clams and 1.17 μM Na/(g dry tissue·hr) for SD clams.

The primary exchange ion for Na is H, although a Na/NH_4 exchange is functional in SD animals. In PW clams, 2.41 μM H/(g dry tissue·hr) is transported in a 1:1 exchange with Na. In SD clams, the net NH_4 flux quadrupled contributing to a Na: $(H + NH_4)$ exchange.

LITERATURE CITED

CAMERON, J. N., 1978. NaCl balance in blue crabs, *Callinectes sapidus*, in fresh water. *J. Comp. Physiol.*, **123**: 127–135.

DIETZ, T. H., 1978. Sodium transport in the freshwater mussel, *Carunculina texasensis* (Lea). *Am. J. Physiol.*, **235**: R35–R40.

DIETZ, T. H., 1979. Uptake of sodium and chloride by freshwater mussels. *Can. J. Zool.* **57**: 156–160.

DIETZ, T. H., and R. H. Alvarado, 1970. Osmotic and ionic regulation in *Lumbricus terrestris*, L. *Biol. Bull.*, **138**: 247–261.

DIETZ, T. H., and R. H. ALVARADO, 1974. Na^+ and Cl^- transport across gill chamber epithelium of *Rana catesbeiana* tadpoles. *Am. J. Physiol.*, **226**: 764–770.

DIETZ, T. H., and W. D. BRANTON, 1975. Ionic regulation in the freshwater mussel, *Ligumia subrostrata* (Say). *J. Comp. Physiol.*, **104**: 19–26.

DIETZ, T. H., and W. D. BRANTON, 1979. Chloride transport in freshwater mussels. *Physiol. Zool.*, **52**: 520–528.

EHRENFELD, J., 1974. Aspects of ionic transport mechanisms in the crayfish *Astacus leptodactylus*. *J. Exp. Biol.*, **61**: 57–70.

FILICE, F. P., 1958. Invertebrates from the estuarine portion of San Francisco Bay and some factors influencing their distribution. *Wasmann J. Biol.*, **16(2)**: 159–211.

FYHN, H. J., AND J. D. COSTLOW, 1975. Anaerobic sampling of body fluids in bivalve molluscs. *Comp. Biochem. Physiol.*, **52A**: 265–268.

GAINEY, L. F., 1978a. The response of the Corbiculidae (Mollusca: Bivalvia) to osmotic stress: the organismal response. *Physiol. Zool.*, **51**: 68–78.

GAINEY, L. F., 1978b. The response of the Corbiculidae (Mollusca: Bivalvia) to osmotic stress: the cellular response. *Physiol. Zool.*, **51**: 79–91.

GAINEY, L. F., AND M. J. GREENBERG, 1977. The physiological basis of the molluscan species abundance-salinity curve: a speculation. *Marine Biol.*, **40**: 41–49.

GARCIA ROMEU, F., AND J. EHRENFELD, 1975. *In vivo* Na^+ and Cl^- independent transport across the skin of *Rana esculenta*. *Am. J. Physiol.*, **228**: 839–843.

GARCIA ROMEU, F., A. SALIBIAN, AND S. PEZZANI-HERNANDEZ, 1969. The nature of the *in vivo* sodium and chloride uptake mechanisms through the epithelium of the Chilean frog, *Calyptocephalella gayi* (Dum et Bibr., 1841). *J. Gen. Physiol.*, **53**: 816–835.

GREENAWAY, P., 1970. Sodium regulation in the freshwater mollusc *Limnaea stagnalis* (L.) (Gastropoda: Pulmonata). *J. Exp. Biol.*, **53**: 147–163.

HAAS, F., 1969. Family Unionidae. Pp. 411–463 in R. C. Moore, Ed., *Treatise on Invertebrate Paleontology*, Pt. N, Geological Society of America, Boulder, Colorado.

HAYASHI, Y., 1956. On the variation of *Corbicula* due to environmental factors. *Venus*, **19**: 54–60.

KEEN, M., AND R. CASEY, 1969. Family Corbiculidae. Pp. 665–669 in R. C. Moore, Ed., *Treatise on Invertebrate Paleontology*, Pt. N, Geological Society of America, Boulder, Colorado.

KERSTETTER, T. H., L. B. KIRSCHNER, AND D. D. RAFUSE, 1970. On the mechanisms of sodium ion transport by the irrigated gills of rainbow trout (*Salmo gairdneri*). *J. Gen. Physiol.*, **56**: 342–359.

KIRSCHNER, L. B., L. GREENWALD, AND T. H. KERSTETTER, 1973. Effect of amiloride on sodium transport across body surfaces of freshwater animals. *Am. J. Physiol.*, **224**: 832–837.

KROGH, A., 1939. *Osmotic Regulation in Aquatic Animals.* Cambridge University Press, London.

MAETZ, J., 1973. Na^+/NH_4^+, Na^+/H^+ exchanges and NH_3 movement across the gill of *Carassius auratus*. *J. Exp. Biol.*, **58**: 255–275.

MAETZ, J., AND F. GARCIA ROMEU, 1964. The mechanism of sodium and chloride uptake by the gills of a freshwater fish, *Carassius auratus* II. Evidence for NH_4^+/Na^+ and HCO_3^-/Cl^- exchanges. *J. Gen. Physiol.*, **47**: 1209–1227.

MAETZ, J., P. PAYAN, AND G. DERENZIS, 1976. Controversial aspects of ionic uptake in freshwater animals. Pp. 77–92 in P. S. Davies, Ed., *Perspectives in Experimental Biology*, Vol. I, Pergamon Press, New York.

MANGUM, C. P., J. A. DYKENS, R. P. HENRY, AND G. POLITES, 1978. The excretion of NH_4^+ and its ouabain sensitivity in aquatic annelids and molluscs. *J. Exp. Zool.*, **203**: 151–157.

MANGUM, C. P., S. U. SILVERTHORN, J. L. HARRIS, D. W. TOWLE, AND A. R. KRALL, 1976. The relationship between blood pH, ammonia excretion and adaptation to low salinity in the blue crab *Callinectes sapidus*. *J. Exp. Zool.*, **195**: 129–136.

MCCORKLE, S., T. C. SHIRLEY, AND T. H. DIETZ, 1979. Rhythms of activity and oxygen consumption in the common pond clam, *Ligumia subrostrata* (Say). *Can. J. Zool.*, **57**: 1960–1964.

POTTS, W. T. W., 1954. The inorganic composition of the blood of *Mytilus edulis* and *Anodonta cygnea*. *J. Exp. Biol.*, **31**: 376–385.

PROSSER, C. L., 1973. *Comparative Animal Physiology.* W. B. Saunders, Philadelphia.

SAINTSING, D. G., AND D. W. TOWLE, 1978. $Na^+ - K^+$ ATPase in the osmoregulating clam, *Rangia cuneata*. *J. Exp. Zool.*, **206**: 435–442.

SHAW, J., 1959. The absorption of sodium ions by the crayfish, *Astacus pallipes* Lereboullet I. The effect of external and internal sodium concentrations. *J. Exp. Biol.*, **36**: 126–144.

SOLORZANO, L., 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnol. Oceanogr.*, **14**: 799–801.

USSING, H. H., 1949. The distinction by means of tracers between active transport and diffusion. *Acta Physiol. Scand.*, **19**: 43–56.

USSING, H. H., D. ERLIJ, AND U. LASSEN, 1974. Transport pathways in biological membranes. *Ann. Rev. Physiol.*, **36**: 17–49.

Reference: *Biol. Bull.*, **159**: 337–348. (October, 1980)

SPECIFICITY IN THE ASSOCIATION BETWEEN *HYDRACTINIA ECHINATA* AND SYMPATRIC SPECIES OF HERMIT CRABS[1]

NEIL A. MERCANDO AND CHARLES F. LYTLE

Department of Biology, The Pennsylvania State University, Ogontz Campus, Abington, Pennsylvania 19001, and Department of Zoology, North Carolina State University, Raleigh, North Carolina 27650

Symbiotic associations between hermit crabs and cnidarians, particularly sea anemones, have been widely investigated. From his extensive behavioral and physiological studies of many hermit crab-sea anemone associations, Ross (1960, 1974) identified varying degrees of specificity, ranging from intimate obligatory relationships, *e.g.*, that between *Pagurus prideauxi* and its "cloak anemone," *Adamsia palliata;* to non-obligatory, possibly chance associations. Since the work of Schijfsma (1935, 1939), the association between hermit crabs and the colonial hydrozoan, *Hydractinia echinata*, has received only limited attention.

Specimens of *Hydractinia echinata* form an encrusting, spinous, mat-like covering on their usual substrate, namely, gastropod shells. The occurrence of hydrozoans (*e.g.*, *Hydractinia*) on hermit-crab-occupied shells influences the ecology and behavior of the host hermit crabs (Jensen, 1970; and Grant and Ulmer, 1974). In their studies of hermit crab populations from the Gulf of Mexico, Wright (1973), Conover (1976), Fotheringham (1976), and Mills (1976a) all discuss the differential occurrence of hydractiniids on shells occupied by *P. longicarpus, P. pollicaris*, and *Clibanarius vittatus*. Mills (1976a) states that smaller, common, sympatric hermit crab species (*e.g., P. annulipes*) "undoubtedly utilize some of the same shells as young individuals of the other three [larger] species," and that "the extent of their participation in the association has not been studied." Although Grant and Ulmer (1974) and Fotheringham (1976) have suggested that the association plays an important role in partitioning the gastropod shell resource, additional work is needed to substantiate and explain this.

Hermit crabs exhibit distinct behavior patterns in selecting shells (Reese, 1962, 1963). Most studies involving selection of shells have focused on the importance of such shell characteristics as species, morphology, weight, volume, aperture size, or a combination of these factors (Reese, 1962, 1963; Markham, 1968; Childress, 1972; Kuris and Brody, 1976; and Conover, 1978). Various aspects of shell availability and utilization as well as intra- and interspecific competition for suitable shells have been described by Orians and King (1964), Vance (1972a, b), Hazlett (1974), Kellogg (1976), Spight (1977) and Scully (1979). As recognized by Reese (1969), the shell functions as a microhabitat of the crab, and consequently, shell selection by hermit crabs should be regarded as a special form of habitat selection.

The purposes of this study were 1) to investigate the degree of specificity between species of hermit crabs and *Hydractinia;* 2) to determine the seasonality of the association; 3) to determine any pattern of specificity in shell-selection behavior of the sympatric hermit crabs *Pagurus annulipes*, *P. brevidactylus*, *P. longicarpus*, and *P. pollicaris*; and 4) to consider the occurrence of *Hydractinia* as a factor in the crabs' partitioning of the gastropod shell resource.

---

[1] Paper No. 6574 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, N. C.

MATERIALS AND METHODS

Four to six samples were obtained each month from November, 1971, to February, 1973, from an area of Bogue Sound southwest of the Morehead City, North Carolina, port turning basin. Using a 1-m scallop dredge covered with 1/4-in mesh netting, collections from the oyster shell substrate were made at depths varying from 3 to 5 m after dredging for 15 min at a constant speed. From the contents of each dredge haul, all gastropod shells were collected and placed in buckets of sea water for later identification and analysis. For each sample, the numbers of unoccupied, snail-occupied, and hermit-crab-occupied shells were recorded. The species of shell and hermit crab also were identified. All shells were examined with a dissecting microscope for the presence of *Hydractinia* polyps.

Behavioral studies of three hermit crab species (*Pagurus annulipes*, *P. longicarpus*, and *P. pollicaris*) were conducted in the laboratory. All animals and shells were maintained in filtered sea water at 22°–24°C, 34–35‰ salinity, and under a 16-hr light: 8-hr dark photoperiod. Before testing, all crabs were removed from their shells and held separately in plastic divider-boxes for at least 1 week. Crabs were evicted by removing the shell apex with wire cutters and inserting a thin piece of wire that was wiggled until the crab vacated the shell. Only crabs possessing all appendages and free of obvious external parasites and injuries were used. Crabs were fed shrimp pellets for 1–2 hr on alternate days. The compartments were then cleaned and refilled with sea water.

Illynassa obsoleta shells, the most common shell species for all crab species, were maintained separately in sea water. The interior of each shell used was cleaned by repeated brushing with wire pipe cleaners and rinsing. The shell exteriors without *Hydractinia* colonies were cleaned by light brushing under flowing tap water. Only shells completely encrusted with a living mat of *Hydractinia* were used as *Hydractinia* shells (HS), and only those without the hydroid and free of other fouling organisms were used as plain shells (PS). The *Hydractinia* colonies were fed freshly hatched *Artemia* larvae for 1–2 hr on alternate days. Their compartments were then cleaned and refilled with sea water.

Due to a shortage of appropriate *Hydractinia*-covered shells and the scarcity of the hermit crab *P. brevidactylus*, this species was not included in the laboratory studies. (While this report was in press we learned of a paper by McLaughlin, 1975, which indicates that the crab identified in our report as *Pagurus brevidactylus* has been described as a new species, *Pagurus carolinensis*.)

Considerable evidence (Reese, 1962, 1963; Vance, 1972a; Kellogg, 1976) indicates that hermit crabs exhibit shell-size specificity. From his extensive studies on shell utilization by hermit crabs, including the species in this study, Kellogg (1971) has shown that the length of the anterior shield (ASL) of the hermit crab carapace is a reliable measure of crab size. The anterior-shield length (the distance from the top of the rostrum to the midpoint of the cervical suture) was measured (± 0.01 mm) with a dissecting microscope fitted with an ocular micrometer. Kellogg (1971) also indicated that shell width and shell weight were reliable measures of shell size. We froze specimens of *P. annulipes*, *P. longicarpus*,

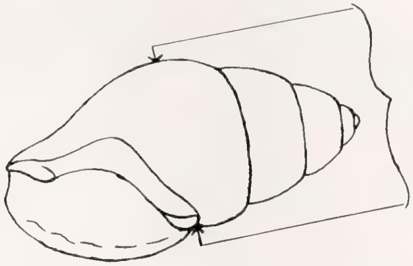


FIGURE 1. Shell width (SW) measurement of gastropod shell size.

and *P. pollicaris* occupying *I. obsoleta* shells that were not fouled, damaged, or covered with *Hydractinia*. Upon thawing, the crabs were easily extracted intact, and ASL measurements were made. Shell widths and shell weights (wet) were obtained for each vacated shell. Between 14 and 30 observations were made for each of the three crab species. The relationships between ASL-shell width and ASL-shell weight were established by standard regression methods. For all three crab species, shell width accounted for more of the variation in crab size than did shell weight. Therefore, shell width (SW) was selected as the primary variate of interest and used in matching suitably sized shells to crabs to be tested. The equations describing the SW-ASL relationship for each crab species are: for *P. annulipes*, $SW = 4.815 + 1.602\ (ASL)$, for *P. longicarpus*, $SW = 1.486 + 2.836\ (ASL)$; and for *P. pollicaris*, $SW = 5.812 + 1.545\ (ASL)$.

Shell width (SW) was defined as the distance between the juncture of the outer lip and first suture on one side of the shell and the point first contacted by calipers on the body whorl directly opposite (Fig. 1). This measurement was always made perpendicular to the longitudinal axis of the shell, *i.e.*, from the shell apex to the aperture of the anterior siphonal canal. Shell width measurements were with vernier calipers graduated in 0.1 mm and read with the aid of a dissecting microscope.

The shell-selection experiments were conducted in an illuminated test chamber where thirty 150-ml culture dishes each containing 100 ml of filtered sea water, were arranged in five rows of six dishes each. With the remainder of the room in darkness, the crabs could be observed through viewing ports with little risk of disturbing them. Into each of these dishes containing 100 ml of filtered sea water a naked (without a shell) hermit crab of known size was placed. Two crabs of each species were randomly selected and assigned within each row. Empty gastropod shells were added after 24 hrs acclimation.

In Experiment I (HS *vs.* PS) 30 individuals of each crab species were tested for preference for *Hydractinia* shells (HS) or plain shells (PS). For each crab, a pair of *I. obsoleta* shells, one covered with a *Hydractinia* colony and one without, was selected according to the shell width (SW) appropriate for the ASL of the crab. Attempts were made to match the shells as closely as possible regarding SW (± 0.05 mm), color, and condition, so that the only noticeable difference between the matched shells would be the presence or absence of a colony of *Hydractina*. Shells were matched to within ± 0.3 mm of the expected acceptable shell size for the crab to be tested. The paired shells were added simultaneously to each dish in a row within the test chamber. The shell occupied after 24 hr was considered the "preferred" shell.

TABLE I

Hermit crab abundance and the utilization of Hydractinia-*shells: HS = shells with* Hydractinia, *and PS = shells without* Hydractinia. ** Based on the overall occurrence of* Hydractinia-*covered shells in the total hermit crab population.*

Hermit crab species	% of total	PS	HS	% HS	χ^2	*P**
Pagurus longicarpus	36.2	700	288	29.1	48.8	<0.001
P. annulipes	28.7	780	3	0.4	191.0	<0.001
P. pollicaris	18.6	257	251	49.4	268.4	<0.001
P. brevidactylus	14.6	397	3	0.8	94.0	<0.001
Paguristes hummi	1.2	30	2	13.7	1.4	>0.100
Clibanarius vittatus	0.4	8	2			
Petrochirus diogenes	0.3	5	3			
Paguristes tortugae	<0.1	1	0			
Total		2178	552	20.2		

In Experiment II (DS *vs.* PS), the same criteria for shell pairing and matching to crabs were employed as in Experiment I. However, shells covered with the perisarcal crust of a dead *Hydractinia* colony (DS) were substituted for shells covered with a living hydroid colony. No crabs used in Experiment I were retested in Experiment II. The observations on each species of crab were made in the same manner as described for Experiment I.

To indicate the degree of "rejection" of shells not chosen in Experiments I and II, a second pair of experiments was conducted. Experiment III was conducted to determine if crabs that rejected HS shells in Experiment I would accept a *Hydractinia* shell when given no other shell to enter. Experiment IV tested whether crabs that rejected a perisarc shell (DS) in Experiment II would accept such a shell when given no other to enter. In these experiments crabs that chose a PS shell in Experiments I or II were evicted and returned naked to culture dishes containing new sea water. After a 24-hr acclimation period, the crabs from Experiment I were retested given only the original HS shell to enter. Similarly, in Experiment IV crabs that rejected a DS shell were given only the original DS shell to enter.

RESULTS

Utilization of gastropod shells by hermit crabs

Eight species of hermit crabs occupied 46% of all gastropod shells collected from Bogue Sound. Of the remaining shells, 32% were unoccupied (*i.e.*, did not contain a hermit crab or snail), and 22% housed a living snail. The four most common species of hermit crabs were *Pagurus annulipes, P. brevidactylus, P. longicarpus,* and *P. pollicaris,* which together comprised over 98% of the 2730 individuals obtained (Table I).

Twenty-three species of gastropod shells were recorded from the total of 5885 shells collected in 78 dredge hauls, with 10 species comprising over 96% of the shells (Table II). Only six shell species, however, were inhabited by all four common crab species. Each of these shell species (*Eupleura caudata, Fasciolaria hunterii, I. obsoleta, Nassarius vibex, Terebra dislocata,* and *Urosalpinx*

TABLE II

Shell utilization and the occurrence of Hydractinia *on common shell species occupied by hermit crabs. PS = shells without* Hydractinia. *HS = shells with* Hydractinia.

Shell species	*P. annulipes*			*P. brevidactylus*			*P. longicarpus*			*P. pollicaris*		
	PS	HS	% HS	PS	HS	% HS	PS	HS	% HS	PS	HS	% HS
Anachis spp.	36	0	0.0	7	0	0.0	—	—				
Busycon spp.	—		—	1	0	0.0	3	0	0.0	58	49	45.8
Eupleura caudata	42	0	0.0	16	0	0.0	9	1	10.0	3	0	0.0
Fasciolaria hunterii	3	0	0.0	2	0	0.0	10	6	37.5	34	46	57.5
Ilyanassa obsoleta	337	1	0.3	241	1	0.4	483	223	31.6	87	71	44.9
Littorina irrorata	—	—	—	—	—	—	18	10	35.7	3	6	66.7
Nassarius vibex	242	2	0.8	55	2	3.6	106	27	20.3	7	1	12.5
Polinices duplicatus		-	—	—			14	6	30.0	36	66	64.7
Terebra dislocata	31	0	0.0	6	0	0.0	6	1	14.3	0	1	100.0
Urosalpinx cinera	77	0	0.0	65	0	0.0	24	12	33.3	17	4	19.1
Other	12	0	0.0	4	0	0.0	21	2	8.7	12	7	36.8
Total	780	3	0.4	397	3	0.8	700	288	29.1	257	251	49.4

cinerea) also supported colonies of *Hydractinia* and, in total, constituted 86% of the shells occupied by hermit crabs.

Even though many shell species were available, one species, *I obsoleta*, was most frequently utilized by all common hermit crab species in Bogue Sound (Table II). *P. annulipes* primarily inhabited *I. obsoleta* (43.2%) and *Nassarius vibex* (31.2%) shells, whereas *P. brevidactylus* mostly inhabited *I. obsoleta* (60.5%) and *Urosalpinx cinerea* (16.2%) shells. Although *P. longicarpus* inhabited the greatest variety of shell species, it was also primarily found in *I. obsoleta* shells (71.5%). The greatest number of *P. pollicaris* (31.1%) were collected in *I. obsoleta* shells. Larger members of this species, however, were frequently collected in two species of *Busycon*, *Fasciolaria hunterii*, and *Polinices duplicatus* shells.

Occurrence of Hydractinia *on hermit crab-occupied shells*

Nearly all of the *Hydractinia*-colonized shells (98%) were occupied by hermit crabs. The remaining colonized shells were found unoccupied. Chi-square contingency table analysis indicated significant differences in the incidence of *Hydractinia* on shells occupied by different crab species. Since *Hydractinia* colonized 20% of all hermit-crab-occupied shells, it would be expected that approximately 20% of each crab species would occupy a *Hydractinia* shell (Table I) if the association were independent of species. The percentages of *Hydractinia* shells occupied by *P. annulipes* (0.4%) and *P. brevidactylus* (0.8%) were less than expected ($P < 0.001$) while the percentages of *Hydractinia* shells occupied by *P. longicarpus* (29%) and *P. pollicaris* (49%) were greater than expected ($P < 0.001$). In addition, the occurrence of the hydroid with *P. annulipes* was not significantly different ($P < 0.50$) from that with *P. brevidactylus*, while its occurrence with *P. longicarpus* and *P. pollicaris* was significantly different between these species as well as between these and the other two species ($P < 0.001$).

If the occurrence of *Hydractinia* were related to shell species and not to hermit crab species, then the hydroid would be present only on certain shell species without regard to the species of crab inhabiting the shell. Therefore, we compared the occurrence of *Hydractinia* on the six shell species occupied by all four hermit crab species (Tables II, III). The hydroid was found on all six shell species; but more

TABLE III

Occurrence of Hydractinia *on shells occupied by four species of hermit crabs from Bogue Sound: PS = shells without* Hydractinia, *and HS = shells with* Hydractinia. ** Based on the occurrence of HS shells occupied by the total hermit crab population as indicated in each section of the table.*

Hermit crabs	PS	HS	% HS	χ^2	*P**
Pooled results of the six shell species inhabited by all four common hermit crab species					
P. annulipes	732	3	0.4	146.9	<0.001
P. brevidactylus	385	3	0.8	74.3	<0.001
P. longicarpus	638	270	29.7	97.4	<0.001
P. pollicaris	148	123	45.4	148.7	<0.001
Total	1903	399	17.3	467.4	<0.001
Same as above but excluding *Ilyanassa obsoleta* shells					
P. annulipes	395	2	0.5	49.8	<0.001
P. brevidactylus	144	2	1.4	15.6	<0.001
P. longicarpus	155	47	23.3	24.4	<0.001
P. pollicaris	61	52	46.0	123.2	<0.001
Total	755	103	12.0	213.0	<0.001
Occurrence of *Hydractinia* on *I. obsoleta* shells only					
P. annulipes	387	1	0.3	82.6	<0.001
P. brevidactylus	241	1	0.4	58.5	<0.001
P. longicarpus	483	223	31.6	57.7	<0.001
P. pollicaris	87	71	44.9	60.5	<0.001
Total	1148	296	20.5	264.9	<0.001

frequently than expected ($P < 0.001$) on shells occupied by *P. longicarpus* and *P. pollicaris* and less frequently than expected ($P < 0.001$) on shells occupied by *P. annulipes* and *P. brevidactylus* (Table III). Also, *Hydractinia* occurred much more often on shells housing *P. pollicaris* and *P. longicarpus* ($P < 0.001$) than on shells occupied by other species. Its frequency on shells housing *P. annulipes* did not differ from its frequency on *P. brevidactylus* shells ($P < 0.70$).

Since one shell species, *I. obsoleta,* comprised over 54% of all hermit-crab-occupied shells, one might suspect that the pattern of association between hermit crab species and *Hydractinia* could result directly from the abundance of *I. obsoleta* shells. To resolve this question, we analyzed the frequency of *Hydractinia* on those shell species commonly occupied by all four crab species, excluding *I. obsoleta* from the pooled totals. Here again, *P. annulipes* and *P. brevidactylus* inhabited *Hydractinia* shells significantly less often ($P < 0.001$) than did *P. longicarpus* or *P. pollicaris* (Table III). Using contingency tables and Fisher's Exact Probability Test, an analysis of the occurrence of *Hydractinia* on *I. obsoleta* shells alone also showed the same pattern of hermit crab specificity (Table III).

The frequency with which each hermit crab species inhabited *Hydractinia* shells was calculated for each monthly collection to determine seasonal differences. In all monthly collections, *Hydractinia* was rarely found on shells occupied by *P.*

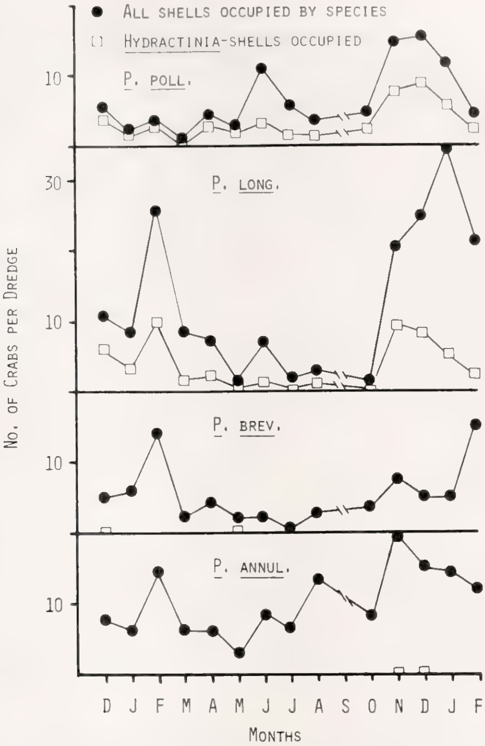


FIGURE 2. Seasonal abundance of *Hydractinia* and species of hermit crabs from Bogue Sound, *P. annul.* = *Pagurus annulipes*, *P. brev.* = *P. brevidactylus*, *P. long.* = *P. longicarpus*, *P. poll.* = *P. pollicaris*.

annulipes or *P. brevidactylus* but was frequently found on shells inhabited by *P. longicarpus* and *P. pollicaris*.

Hermit crabs and *Hydractinia* shells were more abundant from November through February than from March through October (Fig. 2). From November, 1972, to February, 1973, numbers of hermit crabs increased markedly with *P. longicarpus* showing the greatest increase. Concurrent increases in *Hydractinia* shells occupied by *P. longicarpus* and *P. pollicaris* occurred during these months; however, no appreciable increase was evident in *Hydractinia* shells occupied by *P. annulipes* or *P. brevidactylus*.

Shell-selection experiments

Two types of experiments were conducted to determine whether each hermit crab species exhibited shell preference behavior relating to the presence of *Hydractinia* or the perisarcal remains of a dead *Hydractinia* colony. Experiments I and II ("two-choice" experiments) showed that shell preferences exist, while experiments III and IV ("single-choice" experiments) indicated the degree of rejection of a hydroid-covered or perisarc-covered shell.

Experiment I: Hydractinia *shell* (*HS*) *vs. plain shell* (*PS*). In this experiment *P. annulipes* and *P. longicarpus* rejected HS shells more often than expected on the basis of chance and *P. pollicaris* accepted HS shells more often than expected

TABLE IV

Shell selection experiments.

	Two-choice experiments (Random shell selection expected)				Single-choice experiments (100% shell selection expected)			
Hermit Crabs	HS or DS	PS	χ^2	P	HS or DS	NS	χ^2	P
I. *Hydractinia* shell (HS) *vs.* plain shell (PS)					III. *Hydractinia* shell (HS) *vs.* no shell (NS)			
P. annulipes	2	28	22.5	<0.001	14	13	12.5	<0.001
P. longicarpus	8	22	6.5	<0.05	22	0	0	—
P. pollicaris	23	7	8.5	<0.005	7	0	0	—
II. Perisarc shell (DS) *vs.* plain shell (PS)					IV. Perisarc shell (DS) *vs.* no shell (NS)			
P. annulipes	8	12	0.8	>0.05	11	0	—	—
P. longicarpus	9	11	0.2	>0.05	11	0	—	—
P. pollicaris	13	6	2.6	>0.05	3	0	—	—

(Table IV). Only 2 of 30 *P. annulipes* (7%) chose *Hydractinia*-covered shells (HS) after 24 hr. According to the chi-square test, this result is significantly different from random ($P < 0.001$). Twenty-three of 30 (77%) *P. pollicaris*, on the other hand, chose HS shells ($P < 0.005$). Eight of 30 *P. longicarpus* chose HS shells. This result was significantly different from the expected random selection ($P < 0.05$).

Experiment II: Perisarc shell (DS) vs. plain shell (PS). Experiment II paired shells with only the spinous perisarcal crust remaining from a dead *Hydractina* colony (DS) with plain shells (PS). Random shell-selection behavior was exhibited by all three crab species tested in this experiment. Thus, the selection of DS shells as opposed to PS shells by *P. annulipes*, *P. longicarpus*, and *P. pollicaris* was not significant (chi-square, $P > 0.05$). Although only 40% of the *P. annulipes* selected DS shells as compared to 68% of the *P. pollicaris*, this difference was not significant (adjusted chi-square, $P > 0.05$). DS shell selection by *P. longicarpus* also did not differ from that by *P. annulipes* or *P. pollicaris* ($P > 0.05$).

Experiment III: Hydractinia *shell (HS) vs. no shell (NS).* Those individuals rejecting HS shells in Experiment I were retested and exposed only to HS shells. After 24 hr in the test dishes, 13 of the 27 *P. annulipes* remained naked (*i.e.*, without a shell), while 100% of the *P. longicarpus* and *P. pollicaris* entered the *Hydractinia* shell (Table IV). Thus, 48% of the *P. annulipes* did not enter a HS shell, even though it was the only shell available. According to Fisher's Exact Probability Test, HS selection by *P. annulipes* differed significantly from *P. longicarpus* and *P. pollicaris* ($P < 0.02$).

Experiment IV: Perisarc shells (DS) vs. no shell (NS). All crabs tested in this "single-choice" experiment entered the DS shells rather than remain without a shell (Table IV). Therefore, the DS shells did not elicit shell rejection behavior by *P. annulipes* as did the HS shells.

Ten crabs used in shell selection experiments I and II were treated for 24 hr prior to testing in a sea water solution containing 25 ppb $HgCl_2$, to determine if sublethal levels of $HgCl_2$ would alter shell selection behavior. The results indicated no significant difference in the selection of HS or DS shells by crabs dosed with $HgCl_2$ and untreated controls. Fisher's Exact Probability Test was used to determine homogeneity of this data. Since the results for all three hermit crab species showed no significant difference between treated and control animals, these data were pooled.

DISCUSSION

The presence of *Hydractinia* on hermit-crab-occupied shells is clearly not random in Bogue Sound, N. C. The frequency of *Hydractinia*-colonized shells occupied by *P. annulipes* and *P. brevidactylus* was less than 1%, while the frequencies of such shells housing *P. longicarpus* and *P. pollicaris* were 29% and 49%, respectively. This pattern of specificity was significantly different from the expected 20%, the frequency of *Hydractinia* for the total hermit crab population.

Field studies from different areas support the concept of non-random occurrence of hydractiniid hydrozoans on hermit-crab-occupied gastropod shells. Grant and Ulmer (1974) showed that *P. acadianus* inhabits *Hydractinia* shells more frequently than *P. pubescens* in the Frenchman Bay area of Maine. Wright (1973) found that *P. longicarpus* and *P. pollicaris* in Galveston Bay, Texas, often occur in shells supporting either *Hydractinia* or *Podocoryne carnea*, while *Clibanarius vittatus* was rarely observed in shells with hydroids. In other studies of Gulf Coast hermit crabs, Mills (1976a, b) reported 71% of specimens of *P. policaris* and 30% of *P. longicarpus* with either *Hydractinia* or *Podocoryne selena*, and Fotheringham (1976) observed the same two crab species utilizing hydroid-covered shells with a frequency of 62% and 30% respectively. Both of the latter authors found hydractiniid colonies absent or rare on shells occupied by *C. vittatus*. Neither reports any data on *P. annulipes*. We have rarely seen *C. vittatus* in a *Hydractinia*-covered shell along the North Carolina coast.

It is well established that certain species of hermit crabs exhibit clear preferences for the shells of certain gastropod species (Reese, 1962, 1963; Orians and King, 1964; Markham, 1968; and Hazlett, 1971). It has also been suggested that *Hydractinia* frequently colonizes the same species of shells most often occupied by certain crab species (Crowell, 1945). Thus, we must ask whether hermit crabs are selecting for *Hydractinia* or for shell species. If the species of shell were the major factor influencing the association, then certain species of shells commonly occupied by each crab species, especially *P. longicarpus* and *P. pollicaris*, should not be found supporting *Hydractinia* colonies. However, our data shows that those shell species commonly occupied by all four hermit crab species (except *E. caudata*) frequently supported colonies of the hydroid when occupied by *P. longicarpus* and *P. pollicaris*. Conversely, the same shell species rarely supported *Hydractinia* when occupied by *P. annulipes* or *P. brevidactylus*. Similar results by Grant and Ulmer (1974) indicate that the species of shell colonized had little apparent effect on the preference for *Hydractinia* shells exhibited by *P. acadianus*. Their work also suggests that the presence of *Hydractinia* must be an important factor in the selection of shells by certain crab species.

Seasonal factors influence various aspects of the biology of hermit crabs (Reese, 1968; Samuelsen, 1970; and Fotheringham, 1975). The seasonal occurrence of hermit crabs in association with *Hydractinia*, however, has not previously been reported. In each of the monthly collections of this study, *Hydractinia* occurred most frequently on shells occupied by *P. longicarpus* or *P. pollicaris* and rarely on those occupied by *P. annulipes* or *P. brevidactylus*. The former two crab species were most abundant from November through February, when the highest frequencies of *Hydractinia* shells were also found. Thus, the population densities of both *P. longicarpus* and *P. pollicaris* appear to have an important relationship with the abundance of the hydroid.

Various investigators (Jensen, 1970; Grant and Ulmer, 1974; and Conover, 1976) suggest that shell-selection behavior is influenced by the presence of *Hydractinia.* This suggestion is supported by the results of our shell preference studies where *P. annulipes* frequently rejected *Hydractinia* shells even when hydroid-covered shells were the only ones available. On the other hand, *P. longicarpus* and *P. pollicaris* readily accepted *Hydractinia* shells. The rejection of hydroid-covered shells even when no other shells were available was also reported by Wright (1973) for *C. vittatus.* A naked hermit crab that rejected a non-preferred shell in its natural environment would be more vulnerable to predation.

The encrusting mat of perisarc produced by *Hydractinia* gives the shell a rough-textured surface. It might be hypothesized that shell selection or rejection might be due to the crust rather than the living hydroid. Our laboratory experiment showed, however, that each species of hermit crab selected shells without regard to the presence of the perisarcal crust. The living *Hydractinia* colony, therefore, and not the perisarcal crust, appears to provide the stimulus for the differential selection of *Hydractinia* shells by various species of hermit crabs. This conclusion is in accord with the findings of Jensen (1970), who showed that *P. bernhardus* exhibited a clear preference for shells covered with living colonies of *Hydractinia.* We suggest that shell selection behavior of the crabs plays the primary role in determining the pattern of association between symbiotic hydractiniid hydroids and various hermit crab species.

Coexistence of sympatric species depends upon many factors. Vance (1972a) demonstrated that both resource and habitat partitioning are important mechanisms facilitating the coexistence of hermit crab species. The occurrence of *Hydractinia* on available gastropod shells may act as a resource partitioning mechanism, as suggested by Grant and Ulmer (1974) and Fotheringham (1976). Such a mechanism could reduce competition for shells among the sympatric species of hermit crabs from Bogue Sound, N. C.

From his studies of seven sympatric populations of hermit crabs from nearby Beaufort Harbor, N. C., Kellogg (1977) concludes that co-existence of these populations depends upon habitat differences, shell-size partitioning, and shell-species partitioning, in that order. His earlier report (Kellogg, 1976) suggests that the restricted abundance of shells of adequate size and condition may be a factor limiting the size of hermit crab populations, particularly those of the largest species (*P. pollicaris*). The three most abundant species in this area partition the shell resource according to size, with *P. annulipes* commonly occupying the smallest shells, *P. longicarpus* the intermediate, and *P. pollicaris* the largest sizes (Kellogg, 1977). This, however, does not preclude size overlap. In our studies, individuals of each species ranging in ASL size from 1.9 to 2.9 mm were commonly collected. Although smaller shells are more abundant, so are the numbers of species and individuals competing for these shells. Our findings indicate that one species of shell (*I. obsoleta*) is most frequently occupied by each crab species. Shells of this species were most likely not produced in the subtidal habitat of Bogue Sound, since few live gastropods were collected in this area (Mercando, 1975). These factors could only increase competition for shells of small or intermediate size. Since *P. pollicaris* indicates strong preferences for *Hydractinia* shells while *P. annulipes* and *P. brevidactylus* do not, younger members of the former species effectively compete only with *P. longicarpus* for hydroid-covered shells. Therefore, in addition to shell-size selection, *Hydractinia*-shell selection behavior may act to further partition the gastropod shell resource from Bogue Sound.

The authors wish to express their gratitude to: 1) Drs. J. Costlow and W. Kirby-Smith and the staff of the Duke University Marine Laboratory, Beaufort, North Carolina, for advice, assistance and the use of their facilities for most of the field studies; 2) Drs. G. W. Thayer and M. Kjelson, and Mr. M. W. LaCroix and Mr. Nelson Johnson of the National Marine Fisheries Service, Atlantic Estuarine Fisheries Center, National Oceanic and Atmospheric Administration, Beaufort, North Carolina, for their assistance with some of the field collections; and 3) Drs. R. L. Dixon and B. A. Fowler of the Pathologic Physiology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, for the funds, space, and equipment utilized in various aspects of the laboratory phase of this study. Dr. T. Clemmer also provided assistance with statistical problems. We also thank Dr. Thomas G. Wolcott, North Carolina State University, for reviewing the manuscript.

SUMMARY

1. A distinct pattern in the occurrence of *Hydractinia* on shells occupied by four sympatric species of hermit crabs was identified. *Hydractinia* occurred most frequently on shells occupied by *Pagurus longicarpus* and *P. pollicaris* and rarely on shells occupied by *P. annulipes* and *P. brevidactylus.* The abundance of *Hydractinia* fluctuated seasonally, concurrent with changes in the abundance of *P. longicarpus* and *P. pollicaris.*

2. Species of shell appeared to be a less significant factor than species of crab in determining the pattern of association between *Hydractinia* and hermit crabs.

3. In laboratory studies, naked *P. annulipes* rejected *Hydractinia*-covered shells even when offered only such shells to enter, while *P. longicarpus* and *P. pollicaris* readily accepted *Hydractinia*-covered shells. None of the three species showed any preference for or against shells supporting only the perisarcal crust of dead *Hydractinia* colonies.

4. Our results indicate that occurrence of *Hydractinia* on gastropod shells acts to partition this resource and thereby tends to reduce competition among the four sympatric species of hermit crab in Bogue Sound, N. C.

LITERATURE CITED

CHILDRESS, J. R., 1972. Behavioral ecology and fitness theory in a tropical hermit crab. *Ecology,* **53**: 960–964.

CONOVER, M. R., 1976. The influence of some symbionts on the shell-selection behavior of the hermit crabs, *Pagurus pollicaris* and *Pagurus longicarpus. Anim. Behav.,* **24**: 191–194.

CONOVER, M. R., 1978. The importance of various shell characteristics to the shell-selection behavior of hermit crabs. *J. Exp. Mar. Biol. Ecol.,* **32**: 131–142.

CROWELL, S., 1945. A comparison of shells utilized by *Hydractinia* and *Podocoryne. Ecology,* **26**: 207.

FOTHERINGHAM, N., 1975. Structure of seasonal migrations of the littoral hermit crab *Clibanarius vittatus* (Bosc.). *J. Exp. Mar. Biol. Ecol.,* **18**: 47–53.

FOTHERINGHAM, N., 1976. Population consequences of shell utilization by hermit crabs. *Ecology,* **57**: 570–578.

GRANT, W. C., AND K. M. ULMER, 1974. Shell selection and aggressive behavior in two sympatric species of hermit crabs. *Biol. Bull.,* **146**: 32–43.

HAZLETT, B. A., 1971. Influence of rearing conditions on initial shell entering behavior of a hermit crab (*Decapoda, Paguridae*). *Crustaceana,* **20**: 168–170.

HAZLETT, B. A., 1974. Field observations on interspecific agnostic behavior in laboratory reared hermit crabs. *Bull. Mar. Sci.,* **15**: 616–633.

JENSEN, K., 1970. The interaction between *Pagurus bernhardus* (L.) and *Hydractinia echinata* (Fleming). *Ophelia,* **8**: 135–144.

KELLOGG, C. W., 1971. The role of gastropod shells in determining the patterns of distribution and abundance in hermit crabs. Ph.D. Thesis, Duke University, Durham, North Carolina. 210 pp. *Diss. Abst.* Order No. 72-11.101.

KELLOGG, C. W., 1976. Gastropod shells: a potentially limiting resource for hermit crabs. *J. Exp. Mar. Biol. Ecol.,* **22**: 101–111.

KELLOGG, C. W., 1977. Coexistence in a hermit crab species ensemble. *Biol. Bull.,* **153**: 133–144.

KURIS, A. M., AND M. S. BRODY, 1976. Use of principal components analysis to describe the snail shell resource for hermit crabs. *J. Exp. Mar. Biol. Ecol.,* **22**: 69–77.

MARKHAM, J. C., 1968. Notes on growth-patterns and shell-utilization of the hermit crab *Pagurus bernhardus* (L.). *Ophelia,* **5**: 189–205.

MCLAUGHLIN, PATSY A., 1975. On the identity of *Pagurus brevidactylus* (Stimpson) (Decapoda: Paguridae), with the description of a new species of *Pagurus* from the Western Atlantic. *Bull. Mar. Sci.,* **23**: 359–376.

MERCANDO, N. A., 1975. Specificity in the symbiotic association between *Hydractinia echinata* and four species of hermit crabs. Ph.D. Thesis, North Carolina State University at Raleigh, Raleigh, North Carolina. 79 pp. *Diss. Abst.* Order No. 76-14,332.

MILLS, C. E., 1976a. The association of hydractiniid hydroids and hermit crabs, with new observations from North Florida. Pages 467–476 in G. O. Mackie, Ed., *Coelenterate Ecology and Behavior.* Plenum Press, New York.

MILLS, C. E., 1976b. *Podocoryne selena,* a new species of hydroid from the Gulf of Mexico, and a comparison with *Hydractinia echinata. Biol. Bull.,* **151**: 214–222.

ORIANS, G. H., AND C. E. KING, 1964. Shell selection and invasion rates of some Pacific hermit crabs. *Pac. Sci.,* **18**: 297–306.

REESE, E. S., 1969. Behavioral adaptations of intertidal hermit crabs. *Am. Zool.,* **9**: 343–355.

REESE, E. S., 1963. The behavioral mechanisms underlying shell selection by hermit crabs. *Behaviour,* **21**: 78–126.

REESE, E. S., 1968. Annual breeding seasons of three sympatric species of tropical hermit crabs, with a discussion of factors controlling breeding. *J. Exp. Mar. Biol. Ecol.,* **2**: 308–318.

REESE, E. S., 1969. Behavioral adaptations of intertidal hermit crabs. *Am. Zool.,* **9**: 343–355.

ROSS, D. M., 1960. The association between the hermit crab *Eupagurus bernhardus* (L.) and the sea anemone *Calliactis parasitica* (Couch). *Proc. Zool. Soc. London,* **134**: 43–57.

ROSS, D. M., 1974. Evolutionary aspects of associations between crabs and sea anemones. Pages 111–125 in W. B. Vernberg, Ed., *Symbiosis In The Sea.* University of South Carolina Press, Columbia, S. C.

SAMUELSEN, T. J., 1970. The biology of six species of Anomura (Crustacea, Decapoda) from Raunefjorden, Western Norway. *Sarsia,* **45**: 25–52.

SCHIJFSMA, K., 1935. Observations on *Hydractinia echinata* (Flem.) and *Eupagurus bernhardus* (L.). *Arch. Neerlandaises Zool.,* **1**: 261–313.

SCHIJFSMA, K., 1939. Preliminary notes on early stages in the growth of colonies of *Hydractinia echinata* (Flem.). *Arch. Neerlandaises Zool.,* **4**: 93–102.

SCULLY, E. P., 1979. The effects of gastropod shell availability and habitat characteristics on shell utilization by the intertidal hermit crab *Pagurus longicarpus* Say. *J. Exp. Mar. Biol. Ecol.,* **37**: 129–152.

SPIGHT, T. M., 1977. Availability and use of shells by intertidal hermit crabs. *Biol. Bull.,* **152**: 120–133.

VANCE, R. R., 1972a. Competition and mechanism of coexistence in three sympatric species of intertidal hermit crabs. *Ecology,* **53**: 1062–1074.

VANCE, R. R., 1972b. The role of shell adequacy in behavioral interactions involving hermit crabs. *Ecology,* **53**: 1075–1083.

WRIGHT, H. O., 1973. Effect of commensal hydroids on hermit crab competition in the littoral zone of Texas. *Nature,* **241**: 139–140.

Reference: *Biol. Bull.*, **159**: 349–363. (October, 1980)

EFFECTS OF MEDIA WITH LOW SILICIC ACID CONCENTRATIONS ON TOOTH FORMATION IN *ACARTIA TONSA* DANA (COPEPODA, CALANOIDA)

CHARLES B. MILLER,[1] DAVID M. NELSON[1], ROBERT R. L. GUILLARD,[2] AND BONNIE L. WOODWARD[2]

[1] *School of Oceanography, Oregon State University, Corvallis, Oregon 97331, and*
[2] *Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543*

Many calanoid copepods, the dominant herbivores in marine pelagic habitats, have elaborate siliceous teeth set in sockets on the mandibular gnathobase (Beklemishev, 1954, 1959; Sullivan *et al.*, 1975; Vyshkvartseva, 1972). The existence of these siliceous teeth raises the possibility that silicon availability is important for copepod survival, growth, or at least tooth formation. Silicic acid is present in concentrations of 150–175 μM at depth in the Pacific and Indian Oceans and of 40–50 μM in most of the Atlantic Ocean (Armstrong, 1965). However, surface layers of such huge oligotrophic regions as the Sargasso Sea (Menzel and Ryther, 1960) and Central Pacific gyre (SIO Ref. 67–5, 1967) become depleted seasonally to levels as low as 0.3 μM. These levels inhibit diatom growth even in the presence of an excess of other nutrients (Paasche, 1973a; Guillard *et al.*, 1973; Harrison *et al.*, 1976). Therefore, it is possible that other organisms with siliceous parts are inhibited by low silicic acid concentrations as well. If low silicic acid concentration at levels that occur in nature directly inhibits copepod development, then its role in the interaction of phytoplankton and herbivorous zooplankton is complex and requires evaluation.

We have conducted a first study of this possibility, employing the neritic copepod species *Acartia tonsa* Dana. This form is abundant in the waters around Woods Hole from early summer to early fall. It has three siliceous teeth: a curving spine on the ventral end of the tooth list (V), a heavy crown with four rounded points in the center (C_1), and a small bifurcate tooth just to right of center (C_2). The normal form of these teeth is shown in Figure 1. *Acartia tonsa* has been reared by Zillioux and Wilson (1966), Heinle (1969), and others. It tolerates a wide range of food mixtures, container sizes, salinities, and contaminants. We reared *A. tonsa* in media of low silicic acid concentration, trying to demonstrate the level at which development or tooth formation is inhibited.

MATERIALS AND METHODS

Our experimental sequence is shown in Table I. Low silicate media (LSM) were prepared from 1) Sargasso Sea surface water (1.56 μM reactive silicate), and 2) various lots of Vineyard Sound, MA, surface water (1.26–1.56 μM reactive silicate). Water was "stripped" of silicate by growing either *Thalassiosira pseudonana* (Hustedt) Hasle and Heimdal (clone 3H, method of Guillard *et al.*, 1973) or *Phaeodactylum tricornutum* Bohlin (clone Pet Pd, method of D'Elia *et al.*, 1979) in 15- to 18-l lots enriched with nitrate, phosphate, vitamins, and trace metals to f/2 level (Guillard and Ryther, 1962). Incubation was in polyethylene bags ('Cubitainers') under four Sylvania cool white fluorescent lamps on

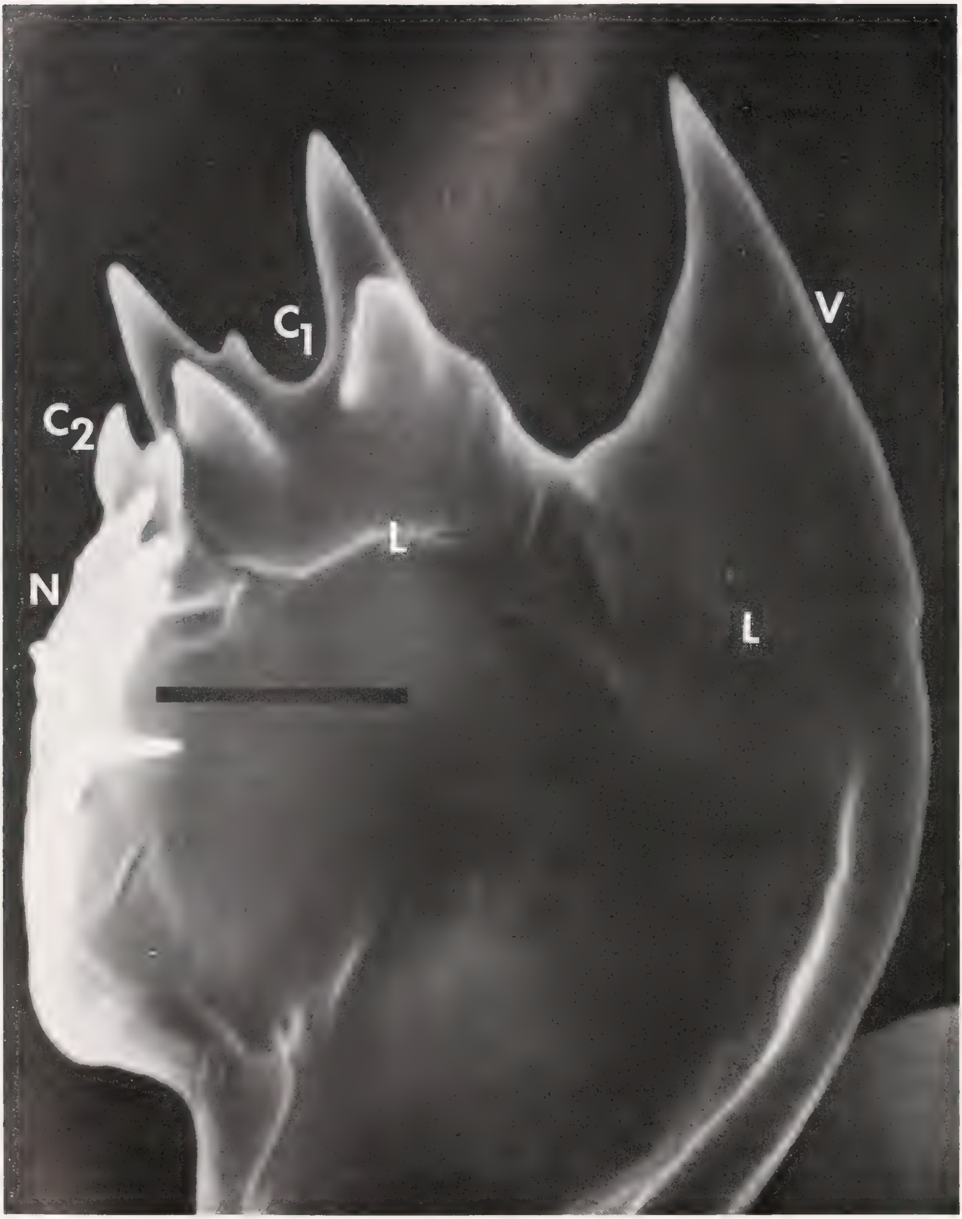
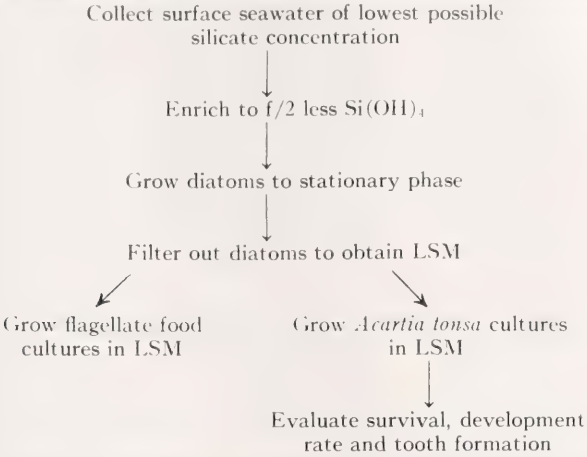


FIGURE 1. Mandibular gnathobase from adult female *Acartia tonsa* reared at 12.6 μM silicic acid (experiment I_2). The black bar represents 10 μm. Symbols: V = ventral tooth, C_1 = large central tooth, C_2 = smaller central tooth, L = limit of siliceous caps, N = nonsiliceous dorsal spine. Scanning electron micrograph by Ann Cornell-Bell (A.C.-B.) using JEOL T20 SEM at 12,500 V accelerating potential.

a 16 hr on: 8 hr off cycle. Temperature was held at 18°C. Increase in cell density of *T. pseudonana* was monitored daily by determining the *in vivo* fluorescence of the suspension with a Turner Designs fluorometer. At the end of exponential growth the cells were filtered from the water with 0.45 μm Millipore

TABLE I

Experimental sequence for producing low silicate medium (LSM) and testing its effects on tooth formation in Acartia tonsa.


Collect surface seawater of lowest possible silicate concentration
Enrich to f/2 less Si(OH)4
Grow diatoms to stationary phase
Filter out diatoms to obtain LSM
Grow flagellate food cultures in LSM
Grow *Acartia tonsa* cultures in LSM
Evaluate survival, development rate and tooth formation

HA filters using plastic filter holders and flasks. Vacuum was limited to 30 mm Hg. Silicic acid concentration in the LSM was determined as "reactive silicate" by the acid molybdate method (Strickland and Parsons, 1972).

P. tricornutum cells were removed when reactive silicate was substantially depleted. This occurred at a high cell density, though less than that at stationary phase. Stripping with *P. tricornutum* produced LSM with 0.08 μM silicic acid. However, this medium was immediately toxic to all three of the food plants used in the copepod rearing. It produced cell lysis. Its pH was found to be 9.5, presumably because of a basic extracellular product of the algae. Vigorous bubbling for several hours with CO_2, reducing the pH to 6.2, followed by bubbling with cotton filtered air raised the pH back to 7.5, removed this toxicity, and increased the silicic acid concentration only slightly. All the food plants and *A. tonsa* grew normally in the LSM treated in this manner. The toxicity is presumably due simply to the high pH, not to the specific base involved. No such effects were noted for LSM produced with *T. pseudonana*, which did not reach such high cell densities and presumably produced less extracellular material.

Three species of flagellated phytoplankton were grown in LSM to serve as food for *A. tonsa* cultures. These were *Isochrysis galbana* Parke (clone ISO), *Dunaliella tertiolecta* Butcher (clone DUN), and *Prorocentrum* sp. (clone EXUV). Each 3–4 days new cultures of each species were started in 450 ml of LSM.

Acartia tonsa was netted from Vineyard Sound on several occasions and placed in cultures by pipetting ten females and four males into 350 ml of water. A mixture of food was added to produce a final total of about 150,000 cells/ml in a volume of 500 ml. Experimental rearing in LSM was done with groups of eggs or very early nauplii produced over 2–3 days in these stock cultures. Some second generation young were used. Each culture used as a source of animals was poured through a 53 μm mesh strainer, rinsed with LSM, and transferred to LSM in a plastic petri dish. Nauplii or eggs were counted under a dissecting microscope

TABLE II

Details of experiments. Replicates have been ordered by subscripts to place similar final silicate concentrations together. Jaws from 15 or more individuals were evaluated from each replicate series, except O, P, and Q where the number of adults and late copepodites was small. "Most" implies approximately 90%.

A. Experiment 1. Sargasso Sea water; 34.5‰ salinity; initially 1.56 μM $Si(OH)_4$; LSM produced with *Thalassiosira pseudonana*; started 20 July; terminated 27 July, 1979; nauplii (N) and copepodites (C) came from different stock containers and were of various ages.

Replicate no.	Starting $[Si(OH)_4]$—μM	Animals	Survivors			Final $[Si(OH)_4]$ μM	Result
			M	F	C		
A_1	0.40 μM LSM	30 N_1 to N_5	4	5	14	0.90	Most jaws with normal teeth, both V and C_1
A_2		40 N_1 to N_5	9	5	9	0.98	
A_3		13 small C, a few N	7	6	4	0.82	
B_1	Control 1: addition to LSM to make 1.5 μM	30 N_1 & N_2		2	19	1.44	Most jaws with normal teeth
B_2		50 N_1 & N_2			46	1.31	
B_3		30 N_1 & N_2			26	—	
C_1	Control 2: unstripped Vineyard Sound water, 1.6 μM	40 N_1 & N_2			35	3.52	Most jaws with normal teeth
C_2		40 N_1 & N_2			30	—	
C_3		42 N_1 & N_2			1	—	

TABLE II—*Continued*

B. Experiment 2. Vineyard Sound water; 33‰ salinity; initially 1.69 μM $Si(OH)_4$; LSM produced with *Thalassiosira pseudonana*; started 6 August; water changed in replicates with subscripts 1–3 14 August; terminated 18 August, 1979. H, J, and K from one stock, I from another.

Replicate no.	Starting $[Si(OH)_4]$–μM	Animals	Survivors			1 week $[Si(OH)_4]$	Final $[Si(OH)_4]$–μM	Result
			M	F	C			
H_1	0.13-LSM	30 N	3	13	1	0.23	0.18	Most jaws with substantial reduction of V and/or C_1. See Fig. 2.
H_2		30 N_1 & N_2	5	15	2	0.33	0.19	
H_3		30 N_1 & N_2	1	8	1	0.18	0.22	
H_4		30 N_1 & N_2	2	12		—	0.26	
H_5		30 N_1 & N_2	4	16	2	—	1.57	Most jaws with normal teeth
I_1	0.13-LSM	30 eggs	1	7	2	0.32	0.27	Most jaws with substantial reduction of V and/or C. See Figs. 3 and 5A. Fig. 5B shows exception.
I_2		30 eggs	1	1	10	0.18	0.21	
I_3		30 eggs		12	3	0.33	0.20	
I_4		30 eggs	3	14	5	—	0.72	
I_5		30 eggs	5	12	5	—	1.27	About half of jaws with some reduction of V or C_1.
J_1	Control 1: addition to LSM to make *ca.* 6 μM	30 N_1 & N_2	3	10	3	6.43	10.21	Most jaws with normal teeth. See Fig. 1.
J_2		30 N_1 & N_2	2	10		6.93	12.55	
J_3		30 N_1 & N_2	3	7	3	5.91	11.65	
J_4		30 N_1 & N_2	2	8	7	—	6.32	
J_5		30 N_1 & N_2	3	14		—	11.02	
K_1	Control 2: unstripped Vineyard Sound water, 1.7 μM	30 N_1 & N_2		2	11	2.94	1.90	Most jaws with normal teeth
K_2		30 N_1 & N_2	1	1	8	3.11	2.38	
K_3		30 N_1 & N_2			12	3.11	2.59	
K_4		30 N_1 & N_2		1	15	—	10.93	
K_5		no count, N_1 & N_2		5	28	—	16.15	

TABLE II—*Continued*

C. Experiment 3. Vineyard Sound water; 33‰ salinity; initially 1.49 μM $Si(OH)_4$; LSM produced with *Phaeodactylum tricornutum*; started 13 August; terminated 24 August 1979; L, M, N in 130 ml; O, P, Q in 400 ml polycarbonate bottles.

Replicate no.	Starting $[Si(OH)_4]$-μM	Animals	Survivors			Final $[Si(OH)_4]$-μM	Result
			M	F	C		
L_1	0.06-LSM	30 N_1 & N_2	3	1	21	0.22	All adult and most copepodite jaws with substantial reduction of V and/or C_1.
L_2		30 N_1 & N_2	3	2	10	0.21	
L_3		30 N_1 & N_2	2	1	16	0.39	About half of jaws with some reduction of V or C_1, in no case both.
L_4		30 N_1 & N_2			3	0.47	
L_5		30 N_1 & N_2	2		28	0.69	
M_1	Control 1: addition to LSM to make *ca.* 5 μM	30 N_1 & N_2	1	1	16	7.17	Most jaws with normal teeth.
M_2		30 N_1 & N_2		8	29	—	
M_3		30 N_1 & N_2	2	2	16	5.90	
M_4		30 N_1 & N_2	3	3	15	—	
M_5		30 N_1 & N_2		4	15	—	
N_1	Control 2: unstripped Vineyard Sound water, 1.5 μM	30 N_1 & N_2	1	5	19	3.15	Most jaws with normal teeth.
N_2		30 N_1 & N_2	2	1	19	—	
N_3		30 N_1 & N_2	1	5	15	4.31	
N_4		30 N_1 & N_2	2	2	23	—	
N_5		30 N_1 & N_2	4	3	19	—	
O_1	0.06-LSM	30 eggs			8	0.16	Most jaws with substantial reduction of V and/or C_1. See Fig. 4.
O_2		30 N_1 & N_2	1	3	12	0.21	
O_3		30 eggs			3	0.26	
P_1	Control 1: addition to LSM to make *ca.* 10 μM	30 N_1 & N_2		2	11	11.2	Most jaws with normal teeth.
P_2		30 eggs			4	—	
P_3		30 eggs	none			—	
Q_1	Control 2: unstripped Vineyard Sound water, 1.5 μM	30 N_1 & N_2		2	12	—	Most jaws with normal teeth.
Q_2		30 eggs			7	4.25	
Q_3		30 eggs	1	1	19	—	

as they entered a small pipette and were placed in polymethyl pentene beakers of LSM. These young were fed LSM food. Total cell density was, again, about 150,000 cells/ml. Experiments were done in 500 and 130 ml volumes. One experiment was run in polycarbonate bottles. In some cases the LSM was changed after 1 week. In others development was completed in the initial medium. One set of experimental control animals was reared in LSM with addition of dissolved sodium metasilicate to approximately f/4 levels to test the direct effect of resupplying silicic acid. Another set was reared in unstripped Sargasso Sea or Vineyard Sound water to test the possibility of adverse effects of the stripping process on water quality. Reactive silicate in the water from all containers was measured at the end of the development period.

RESULTS

Table II lists the completed experiments and their results. Initial silicic acid concentrations in LSM were as low as 0.06 μM. In all cases, concentrations increased substantially during the experimental period. The lowest level after rearing was complete was 0.18 μM. There are two likely sources for this silicate: dissolution of bits of diatom silica which passed the filters used in producing the LSM, and dust. Since reactive silicate did not increase in stock containers of LSM, dust is the more probable source.

Specimens of *Acartia tonsa* survived as well in LSM as in the controls, and developed at normal rates. Several control groups lagged behind the LSM groups in development. This is probably not attributable to treatment differences, but to use of different stocks in establishing the groups. There were small variations in age at the start, and since there were different parents, the groups presumably had somewhat different inherent development rates. This is particularly evident in comparison of the I replicates, which started the experimental period as eggs, with the J replicates, which were early nauplii at the start.

Copepodites and adults of *Acartia tonsa* formed teeth at all of the silicic acid levels tested. However, typical specimens from low silicic-acid levels had teeth much lower in relief than those from high silicic-acid levels. This reduction was most evident in specimens from the 0.18 to 0.3 μM concentrations. Most teeth of specimens reared at levels of 0.8 μM and above closely resembled the teeth shown in Figure 1, which were formed at 12.6 μM. Reduction of teeth in specimens reared at low levels was variable both among individuals and among teeth on the same mandible. Various examples are shown in Figures 2, 3, and 4. There were exceptions (about 10–20%) in both directions: high teeth from specimens reared in the media lowest in silicic acid, and reduced teeth from specimens reared in intermediate levels.

In a number of cases the ventral tooth was so reduced that in the scanning electron microscope there appeared to be a hole completely through the siliceous structure to the chitin or tissue underneath. The jaw shown in Figure 2 has a hole of this kind in the low hummock at the position of the ventral tooth. Higher magnification, to 15,000 diameters, did not resolve the character of these holes any better (Fig. 2B). In fact, virtually no well-defined structure was apparent at any magnification anywhere on the surfaces of reduced teeth. The slight grooves apparent on the sides of all teeth were all that could be resolved better at high magnification (see Fig. 4B).

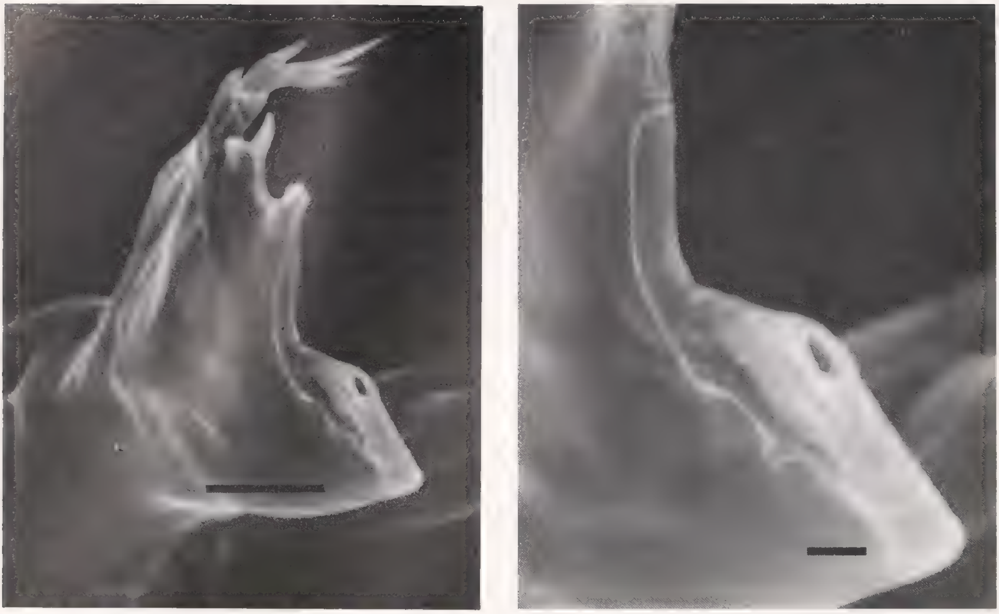


FIGURE 2. A. (Left) Mandibular gnathobase from adult female *A. tonsa* reared at 0.22 μM silicic acid (experiment H_3). The black bar represents 10 μm. The reduced ventral tooth (3 on the reduction rating scale) is on the lower right. B. (Right) Detail of same jaw shows the character of the apparent hole in the ventral tooth. The black bar represents 2.5 μm. SEM by A.C.-B.

By scanning electron microscopy, it was difficult to determine the statistical frequency of reduction. Therefore, a rating scheme was devised using light microscopy of a large sample of the jaws by four observers who did not know the origin of the jaws. The rating scale was 1 for teeth like those of Figure 1 (essentially no reduction), 2 for ventral teeth like that in Figure 3 and central teeth like that in Figure 2 (substantial reduction), and 3 for ventral teeth like those of Figure 2 and central teeth like that in Figure 3 (tooth nearly missing). The sample series was 30 right jaws from female specimens, 10 reared at each of three silicic acid levels. The results are given in Table III. Substantial reduction was found to be primarily and usually present in the specimens from the lowest silicic acid levels tested, about 0.2 μM. While different observers gave different numerical ratings, their agreement on the direction of the differences between specimens was excellent. Using mean ratings for each cell in the tables, combining the mean ratings of 2 and 3 (that is, combining all reduced teeth), and combining the mean ratings for the 0.8 and 10 μM silicic acid levels produced 2×2 contingency tables with sufficiently large expectations for statistical testing. Combining categories after the fact in this way is not strictly legitimate; however, the resulting measure of contingency of tooth reduction on low silicic acid was significant for both teeth ($\chi_1^2 > 10$, $P < 0.01$). Thus the differences between the specimen in Figure 1 and those in Figures 2, 3, and 4 are typical.

Mandibles of stage V copepodites reared in medium of 0.2 μM silicic acid concentration are shown in Figure 5. There is substantial reduction in one specimen, but not in the other. Both the reduction and its variability occur in the younger stages as well as in adults.

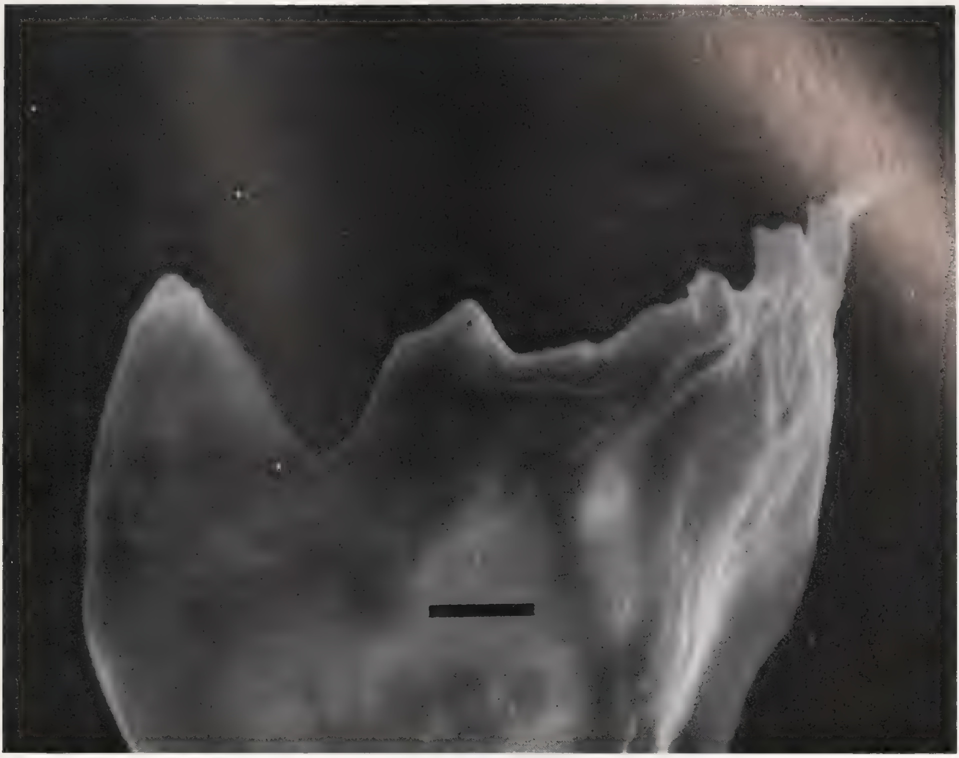


FIGURE 3. Mandibular gnathobase from adult female *Acartia tonsa* reared at 0.27 μM silicic acid (experiment K_1). The central tooth shows severe reduction (3 on the reduction rating scale). The black bar represents 5 μm. The ventral tooth is at left. SEM by Alfred H. Soeldner (A.H.S.) using ISI Mini-SEM.

DISCUSSION

Acartia tonsa successfully extracted silicic acid from concentrations in seawater as low as 0.2 μM and formed it into teeth. On the other hand those teeth were not normal. Formation of normal teeth required concentration of 0.8 μM or above. Thus we must consider both 1) the ability of copepods to draw silicon for tooth formation from lower concentrations and 2) the possible ecologic significance of low silicic acid availability.

Before we conclude that *Acartia tonsa* can draw enough silicon for tooth formation from media with low concentrations of silicic acid, several other possibilities must be considered. It could be that moderate amounts of silicon are present in seawater and LSM which do not react in the acid-molybdate analysis. Silicic acid can form polymers that do not produce the molybdate complex (Alexander, 1953), but which could possibly be used by copepods for tooth formation. However, Burton *et al.* (1970) have done a careful study of this problem. They found "no detectable amount of unreactive silicon in any of the natural water samples analyzed. Polymeric silicon added to the [sea]water samples was unstable and depolymerized completely within a few days." It seems unlikely, therefore, that copepods have any source of silicon in our experiments beside the low levels of reactive silicate which we measure. The result itself argues against another source. Normality of teeth improved with increasing reactive silicate.

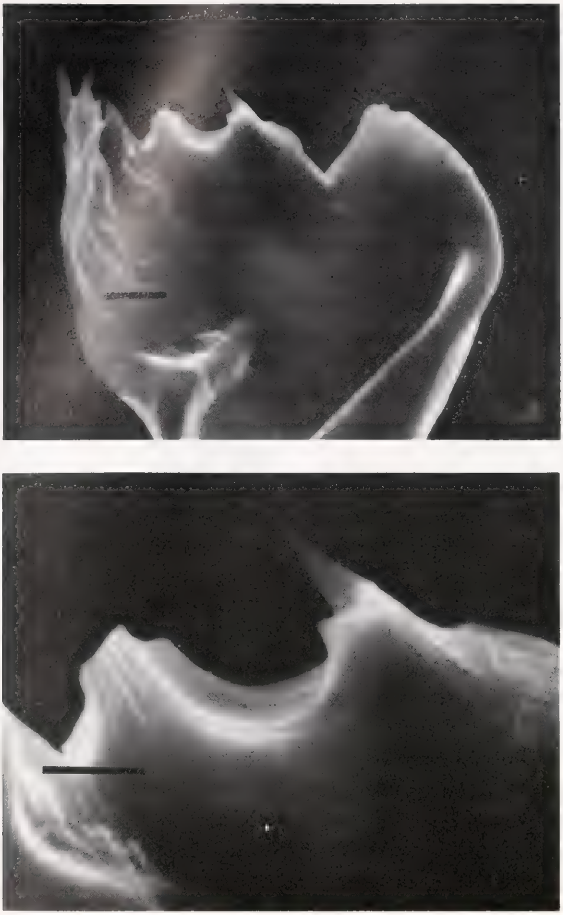


FIGURE 4. A. (Above) Mandibular gnathobase from adult female *A. tonsa* reared at 0.21 μM silicic acid (experiment O_1). Both teeth show substantial, but not severe reduction (2 on the reduction rating scale). The black bar represents 5 μm. The ventral tooth is on the right. B. (Below) Detail of central tooth shows the proximal-to-distal grooves usually on the surfaces of the teeth. The black bar represents 2.5 μm. SEM by A.H.S.

Another possibility is that *Acartia tonsa* eggs might be supplied with sufficient silicon to support tooth formation throughout the life cycle. In that case full depletion of the supply would require several generations. Some calculations show this to be unlikely. Eggs of *A. tonsa* have a diameter of 70 μm and a volume of 1.8×10^5 $\mu m^3 = 1.8 \times 10^{-10}$ liters. If silicic acid were stored in the egg at a concentration equivalent to seawater saturation (*ca.* 1600 μM), then the content of an egg would be about 2.9×10^{-7} μmoles. Plasticene models of the teeth of *A. tonsa* based on SEM pictures like Figure 1 show the volume of the teeth to be approximately $V = 7.2 \times 10^2$ μm^3, $C_1 = 1.7 \times 10^3$ μm^3, and $C_2 = 1.7 \times 10^2$ μm^3. The total tooth volume on both jaws is 5.1×10^3 μm^3. Applying the density of opal (2.1–2.3 gm cm^{-3}) this is equivalent to 1.1×10^{-8} gm. The molecular weight of solid, hydrated silica depends upon the amount of water included. For the formula $SiO_2 + 2H_2O$ the molecular weight is 96 gm $mole^{-1}$, and the amount of silica in the teeth of one adult would be about 1.1×10^{-4}

TABLE III

Results of rating by each of four observers of right jaws from female Acartia tonsa *according to the state of reduction of the ventral (V) and central (C_1) teeth. Rating was on a scale from 1 for no reduction (Fig. 1) to 3 for severe reduction. A. Raw data. The four numbers in each cell are for the four observers. B. Mean numbers of ratings. C. Means combined into 2 × 2 table.*

A.	Ventral tooth, V				Central tooth, C_1		
	Silicic acid levels—μM				Silicic acid levels—μM		
Rating	Low 0.2	Interm. 0.8	High 10–12	Rating	Low 0.2	Interm. 0.8	High 10–12
1	2 | 2 2 | 2	8 | 7 8 | 8	10 | 10 9 | 9	1	3 | 2 2 | 2	9 | 8 7 | 8	9 | 8 10 | 7
2	8 | 8 1 | 7	2 | 2 0 | 1	0 | 0 1 | 1	2	5 | 4 8 | 2	1 | 2 2 | 2	1 | 2 0 | 2
3	0 | 0 7 | 1	0 | 1 2 | 1	0 | 0 0 | 0	3	2 | 4 0 | 6	0 | 0 1 | 0	0 | 0 0 | 1

B.	Ventral tooth, V				Central tooth, C_1		
	Low	Interm.	High		Low	Interm.	High
1	2.00	7.75	9.50	1	2.25	8.00	8.50
2	6.00	1.25	0.50	2	4.75	1.75	1.25
3	2.00	1.00	0.00	3	3.00	0.25	0.25
Mean rating	2.00	1.33	1.05		2.08	1.23	1.18

C.	Ventral tooth, V			Central tooth, C_1	
	Low	Int. + High		Low	Int. + High
1	2.00	17.25	1	2.25	16.50
2 & 3	8.00	2.75	2 & 3	7.75	3.50
		$\chi_1^2 = 12.7$			$\chi_1^2 = 11.0$

μmoles. This is more than the maximum probable content of an egg by a factor of about 380. Additional smaller quantities would have to be present for each copepodite stage, since teeth are lost at each molt. Solid phase silica would have to be present within the egg for the entire life cycle's supply to be contained there. The weak teeth formed in our lowest reactive silicate levels also argue against this possibility. We are certain that *Acartia tonsa* can draw sufficient silicon for tooth formation from media with only slightly more than 0.2 μM silicic acid.

While we have applied a density appropriate for opal in this calculation, we have some evidence that for *Calanus* the siliceous teeth are crystalline. Fragments of tooth have irregular arrays of very sharp spots as their electron diffraction pattern. This is typical of disrupted or somewhat irregular crystals, but not of amorphous opal. Further work on the mineral character of copepod teeth is in progress.

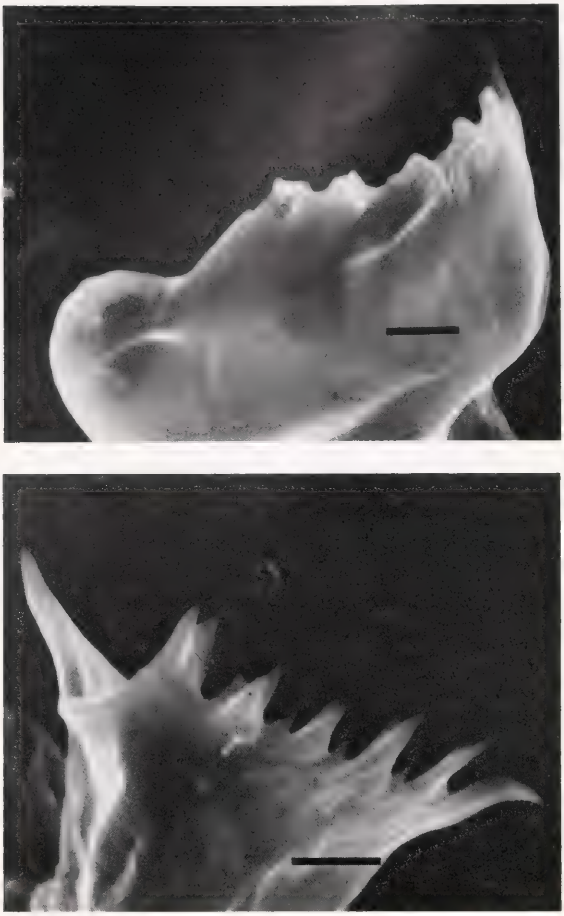


FIGURE 5. A. (Above) Mandibular gnathobase from stage V copepodite of *A. tonsa* reared at 0.21 μM silicic acid (experiment I_2). Both V and C_1 show extreme reduction. The black bar represents 5 μm. Ventral tooth is on left. B. (Below) Mandibular gnathobase from another stage V copepodite reared at 0.21 μM silicic acid (also experiment I_2). Neither V nor C_1 shows much wear. The black bar represents 5 μm. SEM by A.H.S.

A probable explanation for the reduced form of the teeth from LSM is that they have crumbled during use because of insufficient mineralization. The individuals from which all of the jaws in all of the figures came were 1–3 days past their terminal molt when they were preserved for examination. Their teeth had had some time to wear. Figure 5 shows a similar syndrome in stage V copepodites. This stage lasts approximately 1 day at 18°C, and that is apparently sufficient time for substantial wear to occur (if wear is the mechanism of reduction). Alternately, animals reared in LSM may have failed to deposit teeth of normal shape.

Silicic acid concentrations as low as 0.2 μM occur very rarely in the oceans. Thus it seems unlikely that low silicic-acid availability would ever directly inhibit *Acartia tonsa* growth, development, or tooth formation in the field. The approximately 1.5 μM levels encountered in the waters over the continental shelf of the eastern United States that are the habitat of this animal are fully sufficient. A simple observation extends this conclusion to other copepods of pelagic habitats comparably low in silicic acid. We examined a variety of calanoid copepods from surface water at a station in the central Pacific at 30° N, 143° W (samples collected from R/V Alpha Helix in November, 1971). The ambient concentration of reactive silicate was 1.64 μM. Siliceous teeth were found in representatives of all genera studied, except *Candacia*, which has a very reduced mandible. These genera were *Calanus*, *Clausocalanus*, *Paracalanus*, *Scolecithrix*, *Euchaeta*, *Acartia*, *Centropages*, *Pachyptilus*, and *Pontella*. It seems very likely that the capability for making siliceous teeth at silicate levels less than 2 μM is quite general in copepods of oligotrophic habitats.

We performed our experiments with a species obviously accustomed to low silicic-acid levels. Therefore, it may be that we chose a form least likely to demonstrate an inhibition. Perhaps *Acartia clausi* Giesbrecht, which is present in Vineyard Sound from fall to spring, and which does not experience severe silicic-acid depletion in its habitat, will prove more susceptible. Thus, it is still possible that silicic acid availability plays a role in the ecologic succession of copepod species.

The flagellated forms used in our cultures surely present no severe challenge for mastication, even for individuals with the sorts of reduced teeth that develop at low silicic-acid concentration. However, extremely low silicic-acid levels are often found in the field just as a diatom bloom reaches a silicate-limited stationary phase (Dugdale and Goering, 1970; Schelske and Stoermer, 1971; Hafferty *et al.*, 1978). It is possible that copepods undergoing their last maturation molts in such conditions would find themselves unfit for sustained feeding on the abundant diatom food all about them. This could reduce their ability to produce eggs, since most copepods must eat to reproduce, and thus cause a substantial delay in reduction of the bloom. Documenting such an event in a real pelagic ecosystem would require that the investigator be on hand exactly when it occurred, something notably difficult for rare or transient phenomena of all kinds. It is also possible that sufficient silicic acid could be drawn from diatoms in the gut of maturing copeopds to form the teeth for the next phase.

Silicic acid concentrations as low as 0.2 μM, which impair tooth formation in *Acartia tonsa*, are also strongly limiting to silicic acid uptake, silica deposition, and cell division in most planktonic diatoms (Paasche, 1973a, 1973b; Guillard *et al.*, 1973; Azam, 1974; Nelson *et al.*, 1976). With the single known exception of *Phaeodactylum tricornutum* (Lewin *et al.*, 1958), the diatoms have an absolute silicon requirement for growth (Lewin, 1962). Many diatoms can take up silicic acid from concentrations of the order of 1 μM or less. This is in contrast to the silicic-acid uptake capabilities of other algae. It has recently been discovered that many planktonic algae, including *P. tricornutum* and representatives of several major taxa other than diatoms, take up and deposit substantial amounts of silicic acid when it is available (Fuhrman *et al.*, 1978; Bankston *et al.*, 1979). Kinetic experiments with *P. tricornutum* and *Platymonas* sp. (D. Nelson, unpublished data) show their uptake rates to be very low at silicic acid concentrations less than 30 μM. Their uptake systems thus have far less affinity for silicic acid than those of the silicon-requiring diatoms.

We think that this difference in affinity for silicon implies a substantially greater expense in metabolic energy or molecular complexity for systems with high affinity. Only those organisms that strictly require silicon for life meet this expense. The comparability of the silicic acid affinity of *Acartia tonsa* to that of diatoms, in which it is an absolute necessity, thus argues that sound teeth are vitally important for survival in this copepod.

The experiments described were performed by C. B. Miller during the course of a guest investigatorship at Woods Hole Oceanographic Institution in the laboratories of R. R. L. Guillard and Peter H. Wiebe. The work was supported by a grant from the National Science Foundation (OCE-7825762) to Oregon State University and by N.S.F. grant OCE-78-08858 to R.R.L.G. Scanning electron microscopy was done by Ann Cornell-Bell at Marine Biological Laboratory, Woods Hole, and Alfred Soeldner at Oregon State University. We would like to thank Nathanial Corwin, Larry Brand, Steven Boyd, Nancy Marcus, Timothy Cowles, Everett Hogue, William Peterson, and Waldo Wakefield for help. This is contribution No. 4651 from the Woods Hole Oceanographic Institution.

SUMMARY

Acartia tonsa Dana can extract sufficient silicon for formation of siliceous teeth from media with concentrations of silicic acid as low as 0.2 μM. Low silicate media were produced by growing diatoms in seawater collected from oligotrophic habitats and enriched with nutrients other than silicic acid, then removing them by filtration when they reached a silicic acid-limited stationary phase. Most teeth formed by *A. tonsa* in 0.2 μM medium were greatly reduced in profile, probably by increased effects of wear on insufficiently mineralized teeth. Teeth formed at silicic acid levels comparable to those in the field habitat of the copepod (1.5 μM) were normal. The extent of this ability to remove silicon from dilute media is comparable to that of diatoms. It is unlikely that low silicate levels in the field are an important limiting factor for *A. tonsa* or other copepods normally found in oligotrophic, pelagic habitats in the sea.

LITERATURE CITED

Alexander, G. B., 1953. The reaction of low molecular weight silicic acid. *J. Amer. Chem. Soc.*, **75**: 5655–5657.

Armstrong, F. A. J., 1965. Silicon. Pages 409–432 in J. P. Riley and G. Skirrow, Eds., *Chemical Oceanography*. Vol. 1. Academic Press, New York.

Azam, F., 1974. Silicic acid uptake in diatoms studied with $[^{68}\text{Ge}]$ germanic acid as tracer. *Planta* (Berlin), **121**: 205–212.

Bankston, D. C., N. S. Fisher, R. R. L. Guillard, and V. T. Bowen, 1979. Application of dc plasma optical emission spectrometry to studies of element incorporation by marine phytoplankton. *U.S.D.O.E. Environ. Q. Report,* EML **356**: 509–531.

Beklemishev, K. V., 1954. The discovery of siliceous formations in the epidermis of lower crustacea. *Dokl. Akad. Nauk SSSR,* **97**: 543–545.

Beklemshev, K. V., 1959. On the anatomy of masticatory organs of copepoda. Report 2: the masticatory edge in mandibles of certain species of Calanidae and Eucalanidae. *Tr. Inst. Okeanol.*, **30**: 148–155.

Burton, J. D., T. M. Leatherland, and P. S. Liss, 1970. The reactivity of dissolved silicon in some natural waters. *Limnol. Oceanogr.*, **14**: 473–476.

D'Elia, C. G., R. R. L. Guillard, and D. M. Nelson, 1979. Growth and competition of the marine diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana.* I. Nutrient effects. *Marine Biology,* **50**: 305–312.

Dugdale, R. C. and J. J. Goering, 1970. Nutrient limitation and the path of nitrogen in Peru Current production. *Anton Bruun* Report No. 4, Texas A & M Press, pp. 5.3–5.8.

Fuhrman, J. A., S. W. Chiolm, and R. R. L. Guillard, 1978. Marine alga *Platymonas* sp. accumulates silicon without apparent requirement. *Nature,* **272**: 244–246.

Guillard, R. R. L., and J. H. Ryther, 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.*, **8**: 229–239.

Guillard, R. R. L., P. Kilham, and T. A. Jackson, 1973. Kinetics of silicon-limited growth in the marine diatom *Thalassiosira pseudonana* Hasle and Heimdal (*Cyclotella nana* Hustedt). *J. Phycol.*, **9**: 233–237.

Hafferty, A. J., L. A. Codispoti, and A. Huyer, 1978. JOINT-II R/V Melville legs I, II, and IV, R/V Iselin leg II bottle data March 1977–May 1977. *IDOE Coastal Upwelling Ecosystem Analysis Data Report 45,* Univ. Wash., Seattle. 779 pp.

Harrison, P. J., H. L. Conway, and R. C. Dugdale, 1976. Marine diatoms grown in chemostat under silicate or ammonium limitation. I. Cellular chemical composition and steady-state growth kinetics of *Skeletonema costatum. Marine Biology,* **35**: 177–186.

Heinle, D. R., 1969. Culture of calanoid copepods in synthetic seawater. *J. Fish. Res. Bd. Can.,* **26**: 150–153.

Lewin, J. D., 1962. Silification. Pp. 445–455 in R. A. Lewin, Ed., *Physiology and Biochemistry of Algae.* Academic Press, New York.

Lewin, J. D., R. A. Lewin, and D. E. Philpott, 1958. Observations on *Phaeodactylum tricornutum. J. Gen. Microbiol.,* **18**: 418–426.

Menzel, D. W., and J. H. Ryther, 1960. The annual cycle of primary production in the Sargasso Sea off Bermuda. *Deep-Sea Res.,* **6**: 351–367.

Nelson, D. M., J. J. Goering, S. S. Kilham, and R. R. L. Guillard, 1976. Kinetic of silicic acid uptake and rates of silica dissolution in the marine diatom *Thalassiosira pseudonana. J. Phycol.,* **12**: 246–252.

Paasche, E., 1973a. Silicon and the ecology of marine plankton diatoms. I. *Thalassiosira pseudonana* (*Cyclotella nana*) grown in a chemostat with silicate as the limiting nutrient. *Marine Biology,* **19**: 117–126.

Paasche, E., 1973b. Silicon and the ecology of marine planktonic diatoms. II. Silicate-uptake kinetics in five diatom species. *Marine Biology,* **19**: 262–269.

Schelske, C. L., and E. F. Stoermer, 1971. Eutrophication, silica depletion and predicted changes in algal quality in Lake Michigan. *Science,* **173**: 423–424.

Scripps Inst. Oceanogr., 1967. *Data report: physical, chemical and biological data Ursa Major Expedition.* SIO Ref. No. 67–5. 43 pp.

Strickland, J. D. H., and T. R. Parsons, 1972. A practical handbook of seawater analysis, rev. edn. *Bull. Fish. Res. Bd. Can.,* **167**: 1–311.

Sullivan, B. K., C. B. Miller, W. T. Peterson, and A. H. Soeldner, 1975. A scanning electron microscope study of the mandibular morphology of boreal copepods. *Marine Biology,* **30**: 175–182.

Vyshkvartseva, N. V., 1972. Structure of the mandibles in the genus *Calanus* s.l. in relation to latitudinal zonality. Pp. 186–199 in B. E. Bykhovskii and Zh. A. Zvereva, Eds., *Geographical and Seasonal Variability of Marine Plankton.* [Translation ISBN-O-7065-1494-7.]

Zillioux, E. J., and D. F. Wilson, 1966. Culture of a planktonic calanoid copepod through multiple generations. *Science,* **151**: 996–998.

Reference: *Biol. Bull.*, **159**: 364–375. (October, 1980)

ION AND WATER BALANCE OF THE HYPO- AND HYPEROSMOTICALLY STRESSED CHITON *MOPALIA MUSCOSA*

WILLIAM M. MORAN[1] AND RICHARD E. TULLIS

Moss Landing Marine Laboratories, California State Universities, Moss Landing, California; and Dept. of Biological Sciences, California State University Hayward, Hayward, California 94542

Molluscs inhabiting rocky intertidal zones and estuaries often experience salinity stress, as freshwater run-off after heavy rain (Boyle, 1969) or tidal fluctuations of salinity (Stickle and Ahokas, 1975). A rapid reduction of external salinity causes these organisms to gain water osmotically, with the result that physiological functions such as respiration, feeding, and locomotion may be severely impaired (Oglesby, 1975). The water taken up can be considered to enter both the extracellular space (in the case of animals with open circulatory systems, the blood) and the intracellular space. Volume regulation of both is required for survival.

Cell volume regulation has been extensively investigated in marine and estuarine invertebrates (Pierce and Greenberg, 1973). Control of cell volume occurs through changes in the concentration of intracellular free amino acids (FAA). For example, during hyposmotic stress these acids leave the cell intact followed by osmotically obligated water, and cell volume is restored (reviewed by Watts and Pierce, 1978).

Much less is known about whole-animal volume regulation of molluscs. This aspect of volume regulation is complex. It may simultaneously involve salt and water movements between the animal's body fluids and the medium, shifts of free amino acids between cells and extracellular fluids (cell volume regulation), change of osmotic permeability, and change in excretion rates (Fletcher, 1974b). In addition, these mechanisms may contribute to volume control at different times after exposure of the organism to hyposmotic sea water.

Most work on volume regulation of molluscs has been performed with bivalves and gastropods. Both groups have an encompassing shell which makes accurate wet-weight determinations difficult. The polyplacophoran molluscs, the chitons, do not have an encompassing shell. Instead, they have eight serially arranged plates embedded in the girdle on their dorsal surface. In addition, chitons readily attach themselves to petri dishes, facilitating weighing of the animal. The chiton's wet weight is determined by subtracting the weight of the petri dish.

The chiton *Mopalia muscosa* inhabits the rocky intertidal zone of the Pacific coasts of the United States and Canada. The purpose of this study was to characterize whole-animal volume regulation in *M. muscosa*. We also report the blood-ion concentrations and muscle-tissue water content of chitons acclimated to different salinities.

---

[1] Present address: Department of Zoology, University of Maryland, College Park, MD 20742.

Materials and Methods

Collection of animals and maintenance conditions

Specimens of *Mopalia muscosa* were collected on the south jetty of Elkhorn Slough, Moss Landing, California. All chitons were taken within the intertidal zone, and between −0.3 and +0.6 m of mean lower low water. Collections were restricted to this intertidal range to limit intraspecific differences resulting from different habitats. At the laboratory, the chitons were placed in a maintenance tank which held aerated normal sea water (33.3–34.5‰ salinity; pH = 7.7–8.1) at 13°C ± 2°. All chitons were allowed 6–13 days to adjust to these conditions before experiments were performed. The chitons ranged in weight from 5 to 22 g.

Source and preparation of experimental SW

Sea water was obtained from the mouth of Elkhorn Slough at high tide, filtered with a Glass Fiber Type A filter, and approximately 11 g of an artificial sea-salt mixture (Instant Ocean) was added to a liter of filtered sea water (SW) in order to raise the salinity to 42.1‰ (125% SW); lower salinities were made by diluting the 125% SW with deionized water. The salinity of all solutions was determined before use with an induction salinometer (± 0.01‰).

Acclimation of chitons to experimental SW

Most experiments were conducted at 13.3°C; the pH varied between 8.0 and 8.3. The effect of salinity on blood-ion concentrations was examined by acclimating batches of three chitons to experimental media of 125, 100, 75, and 60% SW for 48 hr. Then a sample of hemolymph was removed from each damp-dried chiton by puncturing the body wall medially just beneath the eighth valve with a needle and 1 cc syringe. The pericardium is near the region where hemolymph was obtained. However, pericardial fluid would be expected to be colorless when withdrawn, due to ultra-filtration of hemolymph through the heart wall. In each case blue fluid was obtained, indicating the presence of hemocyanin, and assuring that the fluid was blood and not pericardial fluid. The hemolymph collected from each animal was centrifuged to remove cells. The supernatants were used for ion-concentration determinations.

The effect of hyposmotic SW on the kinetics of change of chiton blood Na$^+$ and Cl$^-$ concentrations was investigated by placing chitons in 60% SW and then removing three chitons at 2, 4, 6, 8, 12, and 24 hr for hemolymph-ion analysis. Three chitons from the maintenance tank served as time 0 samples.

Measurement of SW and blood-ion concentrations

Sodium, K$^+$, Ca^{2+}, and Mg^{2+} concentrations of hemolymph and SW were determined by atomic-absorption spectrophotometry (Perkin-Elmer 305B). Sodium, Ca^{2+}, and Mg^{2+} concentrations were measured by diluting hemolymph and SW appropriately with 1000 ppm K$^+$, in order to prevent ionization effects, in 1% HNO$_3$; the diluting solution for the Ca^{2+} and Mg^{2+} determinations also contained 1% La^{3+} to prevent phosphate interference with Ca^{2+} and Mg^{2+} determinations. The diluting solution for determination of K$^+$ concentrations contained 1000 ppm Na$^+$, again to prevent ionization effects, in 1% HNO$_3$. Standards were prepared with the appropriate diluting solution. Chloride concentrations were determined with an Oxford Titrator by the mercuric titration method of Schales and Schales (1941).

Measurement of wet-weight changes as an indicator of volume regulation

Chitons were placed in the maintenance SW on preweighed plastic petri dishes and allowed 2–3 days to attach to them. The petri dish plus the chiton was weighed and then placed into 2 l of aerated experimental SW and weighed at 1, 2, 4, 6, 8, 12, and 24 hr. Wet weights were determined by removing the petri dish with the attached chiton from the SW, blotting dry, and immediately weighing on an analytical balance to the nearest 0.01 g. Nine replicate weighings of a single chiton in 100% SW gave a standard deviation of ±0.8% body wet weight. The small volume of water trapped in the mantle cavity of the chitons was assumed to remain unchanged during the experiment. The resulting small overestimates of the chiton's true wet weight were too small to seriously affect conclusions about volume regulation.

Lange and Mostad (1967) have criticized volume-regulation experiments using whole animals: They state that weight variations could be caused by excretion and loss of fecal pellets. Few fecal pellets were seen in the containers during volume-regulation experiments with *M. muscosa;* defecation probably did not contribute to the variability in wet-weight determinations. However, excretion remains a possible source of variation.

The effect of temperature on volume regulation was studied by holding the chitons for 2–3 hr in 100% SW at either 7° or 19°C and then placing them in 60% SW of the same temperature and monitoring wet weights at 0, 2, 4, 6, 8, 12, and 24 hr.

Calculations of osmotic permeability (P_{os}) and of chitons as osmometers

Calculations of osmotic permeability, P_{os}, and the theoretical rate of weight change of chitons responding as if they were perfect osmometers were based on the wet weight of the soft parts, determined as follows. Sixteen chitons were blotted dry and their wet weight determined by weighing on an analytical balance. Next, the chitons' plates were removed by scraping all girdle tissue from the plates, and all eight plates were weighed. Then, a regression line of plate wet weight *vs.* total-body wet weight was calculated. The equation was $Y = 0.34 - 0.6X$, and r, the coefficient of correlation, was 0.998. Thus plate wet weight could be obtained from a measurement of total-body weight. The wet weight of the soft parts could then be found by subtracting the plate wet weight from the total-body wet weight.

In order to base all P_{os} values and theoretical values for chitons responding as perfect osmometers on the soft-part water content, the total body water content had to be determined. This was done by drying four chitons to constant weight at 67°C. Using the above regression equation, the soft-part water content of the chitons could be determined.

The osmotic water permeability, P_{os}, was calculated according to Fletcher (1974a):

$$P_{os} = \frac{1}{t(C_1 + C_o' - C_o)} \left[f + \frac{Z_o C_o'}{C_1 + C_o' - C_o} \times \log_e \left\{ 1 - \frac{f(C_1 + C_o' - C_o)}{Z_o(C_o - C_1)} \right\} \right] \quad \text{(Equation 1)}$$

where P_{os} is in kg H_2O/kg animal × hr × unit osmolal concentration difference, C_o is the osmolality of the acclimatization medium (100% SW), C_o' the osmolality of chiton hemolymph in the acclimating medium, Z_o the water content of the animal (based on soft parts) (kg H_2O/kg animal when acclimated to C_o), C_1 is the osmolality of the experimental medium (60% SW) and f is the fractional increase in weight t hrs after transfer to the experimental medium. Whenever the term $C_o' - C_o$ occurred in the equation it was cancelled, since chitons apparently are osmoconformers (McGill, 1975; Simonsen, 1975; Boyle, 1969).

At various times after the chitons were placed in 60% SW, f was measured. P_{os} was calculated for each time, and a graph of P_{os} against t plotted.

The theoretical increase in the weight of soft parts at a given time t of chitons behaving as if they were perfect osmometers was calculated according to Fletcher (1974b):

$$t = \frac{1}{P_{os}(C_1 + C_o' - C_o)} \left\{ \frac{Z_o C_o'}{C_1 + C_o' - C_o} \times \log_e \left[\frac{Z_o(C_o - C_1)}{Z_o(C_o - C_1) - f(C_1 + C_o' - C_o)} \right] - f \right\} \quad \text{(Equation 2).}$$

Initial values of P_{os} must be used to calculate the theoretical osmometer curves; later values would be reduced by the mechanisms regulating chiton volume. The initial values were obtained from the plot of P_{os} against time.

Determination of muscle-tissue water content

After 9–10 chitons had acclimated for 48 hr to the various experimental media, the muscle-tissue water content was determined. Small portions of foot muscle (60–110 mg) were oven dried at 96°C for 20 hr, and the percent water content was obtained from the difference between the wet and dry weights. These data were arcsine transformed for statistical purposes (Sokal and Rohlf, 1969). However, the untransformed data are presented graphically.

Statistical methods

Means of two treatment groups were compared by Student's *t* test after determining homogeneity of variance with an *F* test (Sokal and Rohlf, 1969). In a few cases the non-parametric Mann Whitney *U* test was used to test for statistical differences between two treatment groups. Regression analysis was performed by the method of least squares, and the significance of the regression coefficient was established by a *t* test (Sokal and Rohlf, 1969). Statistical significance is considered to be the 5% level of probability.

Results

Effect of salinity on hemolymph ion concentrations

Following acclimation, the hemolymph Na^+ and Cl^- concentrations of *Mopalia muscosa* were equivalent to the SW concentrations at all salinities tested. Hemolymph K^+ and Mg^{2+} concentrations were also isoionic to the SW, except at 60% SW where the hemolymph K^+ and Mg^{2+} concentrations were greater than the SW values ($P < 0.05$, K^+; $P < 0.05$, Mg^{2+}, Mann Whitney *U* test). The blood Ca^{2+}

TABLE I

Blood ion concentrations of M. muscosa *(mEq/l), at different salinities, compared to the SW concentration. N = 3 for each ion at each salinity. Values are expressed as the mean ±SE.*

	% SW	Blood	Sea Water
Cl^-	125	673.2 ± 10.00	686.2 ± 3.70
	100	531.2 ± 9.10	543.5 ± 0.60
	75	407.5 ± 1.70	402.4 ± 2.10
	60	343.7 ± 3.80	348.1 ± 1.10
Na^+	125	590.8 ± 3.50	604.5 ± 1.00
	100	483.2 ± 13.00	479.2 ± 6.00
	75	374.9 ± 5.10	377.0 ± 8.10
	60	303.4 ± 4.90	318.8 ± 6.00
K^+	125	11.6 ± 0.60	11.0 ± 0.25
	100	10.2 ± 0.50	8.6 ± 0.20
	75	7.3 ± 0.12	6.9 ± 0.05
	60	5.8 ± 0.07*	5.5 ± 0.05
Ca^{2+}	125	27.3 ± 0.50	26.7 ± 0.10
	100	21.8 ± 0.29	20.8 ± 0.01
	75	17.5 ± 0.13**	14.8 ± 0.00
	60	15.4 ± 0.18**	12.7 ± 0.01
Mg^{2+}	125	119.5 ± 2.75	122.9 ± 0.20
	100	96.9 ± 1.18	97.2 ± 1.60
	75	75.2 ± 0.13	75.1 ± 0.20
	60	61.2 ± 0.21***	59.9 ± 0.00

* $P < 0.05$, hemolymph *vs.* SW (*t* test)
** $P < 0.001$, hemolymph *vs.* SW (*t* test)
*** $P < 0.05$, hemolymph *vs.* SW (Mann Whitney *U* test)

concentration of chitons acclimated to 125% and 100% SW were equal to that of SW; but the hemolymph Ca^{2+} concentration was hyperionic in chitons acclimated to 75 and 60% SW (Table I).

Hemolymph Cl^- concentration of chitons transferred to 60% SW from 100% SW reached equilibrium with the 60% SW Cl^- concentration 8 hrs after transfer. Blood Na^+ concentration came into equilibrium between 8 and 12 hr after transfer of chitons from 100 to 60% SW (Fig. 1).

Volume regulation

In the fall, *M. muscosa* regulates volume in salinities as low as 60% SW. Chitons exposed to 60% SW for 6 hr showed a maximum increase of approximately 20% in total-body wet weight, whereas chitons exposed to 75% SW for 2 hr showed a maximum increase of 10%. In both 60 and 75% SW there is a decline of 6–7% total-body wet weight after the maximum weight gain. In addition, the rates of weight loss were equivalent in both hyposmotic salinities (Fig. 2). A regression of maximum weight gain of fall chitons exposed to 60% SW on total-body wet weight showed no correlation between chiton size and maximum weight gain.

Chitons returned to 100% SW following exposure to 60 or 75% SW showed a 3–4% undershoot of the original wet weight during the first hour of exposure

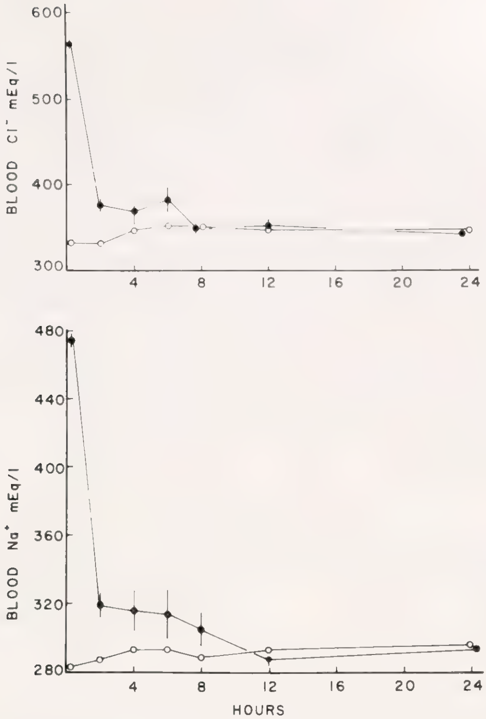


FIGURE 1. Solid circles indicate the effect of time and salinity on blood Na^+ and Cl^- concentration of *M. muscosa*. Chitons were transferred from 100 to 60% SW at time zero. Each point represents the mean, and vertical bars ± 1 SE, of three chitons. Open circles = 60% SW Na^+ and Cl^- concentration.

(Fig. 2). Two to four hr after transfer to 100% SW the undershoot peaked at approximately 6% loss of body weight. Over the next 20 hr a gradual but significant ($P < 0.01$, 60% SW; $P < 0.05$, 75% SW) increase in weight occurred. Chitons exposed to 125% SW lost 8–9% of their original total-body weight and showed limited volume regulation after 12 hr exposure to the hyperosmotic medium.

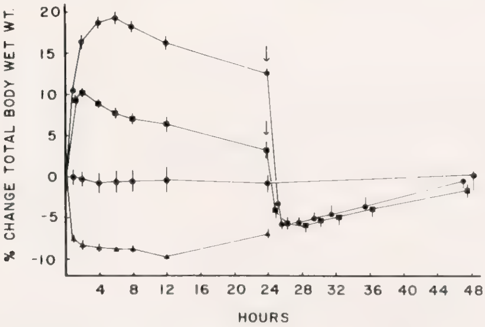


FIGURE 2. Volume regulation of *M. muscosa* in fall at different salinities. Chitons in 60% SW, N = 6 (solid circles); in 75% SW, N = 6 (solid squares); in 100% SW, N = 4 (solid hexagon); in 125% SW, N = 3 (solid triangle). Vertical bars indicate ± 1 SE. At the arrows the chitons exposed to 60 and 75% SW were returned to 100% SW.

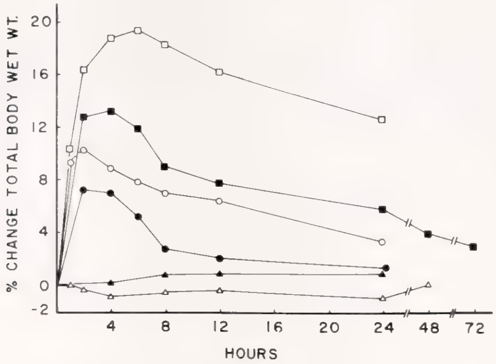


FIGURE 3. Volume regulation of *M. muscosa.* Spring chitons in 100% SW, N = 5 (solid triangles); in 75% SW, N = 7 (solid circles); in 60% SW, N = 5 (solid squares). Fall chitons in 100% SW (open triangles), in 75% SW (open circles) and in 60% SW (open squares). Sample size of fall chitons is shown in Fig. 2. Error bars are omitted for clarity.

Another set of volume regulation experiments were performed over 72 hr to determine if *M. muscosa* was capable of further reducing its weight if specimens spent more than 24 hr in 60% SW. These experiments were done in the spring. An additional weight reduction of only 2% occurred over the next 48 hr (Fig. 3). The volume regulation response of fall chitons is also shown in Figure 3 to illustrate the difference in volume regulation of fall and spring chitons. Fall chitons in both 60 and 75% SW gain significantly ($P < 0.01$, both salinities) more weight than spring animals. In addition, spring chitons regulate volume more quickly than do fall chitons in both 60 and 75% SW.

High temperature, 19°C, did not affect volume regulation of *M. muscosa* in 60% SW. However, low temperature, 7°C, reduced the chitons' ability to regulate volume (Fig. 4). Significant differences between the 7° and 13°C curves occurred at 2 ($P < 0.02$), 8 ($P < 0.05$), and 12 hr ($P < 0.05$). The 6 hr values were not significantly different because of the large amount of variation associated with the 6 hr value of the control curve, 13°C.

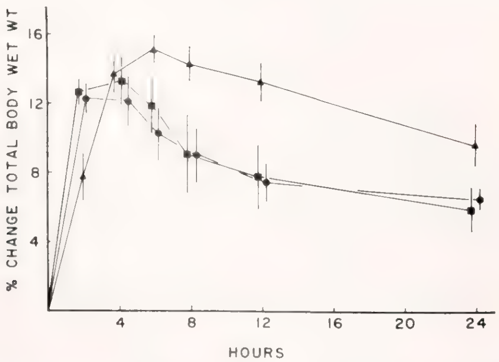


FIGURE 4. Volume regulation of *M. muscosa* in 60% SW at three temperatures in the spring. Chitons at 13°C, N = 5 (solid squares); 7°C, N = 6 (solid triangles); 19°C, N = 6 (solid circles). Data points are offset on the ordinate for clarity of diagram.

Osmotic permeability and chitons responding theoretically as osmometers

The osmotic permeability, P_{os}, (calculated according to equation 1) was 0.71 ± 0.045 kg H_2O/kg animal × hr^{-1} × unit osmolal concentration difference after 1 hr of exposure of chitons to 60% SW; the 2 hr value was 0.66 ± 0.10. These P_{os} values did not differ significantly and were averaged and used to calculate the theoretical rate of weight change (equation 2) of chitons responding as if they were perfect osmometers.

M. muscosa does not respond as an osmometer in hyposmotic SW. The per cent increase of soft-part wet weight of chitons behaving as if they were simple Boyle-van't Hoff osmometers is 46.4%; this value agrees well with the asymptotic value of 48.7% calculated according to equation 2. The curve of chitons responding as if they were osmometers and the curve of observed weight change begin to separate after approximately 1 hr. Therefore, mechanisms of volume control are coming into effect within 1 hr after transfer of chitons to 60% SW (Fig. 5).

Muscle tissue water content

With acclimation (2 days) to decreasing salinity the foot-muscle tissue of *M. muscosa* becomes increasingly hydrated. However, this tissue does not appear to respond as a Boyle-van't Hoff osmometer ($P < 0.001$, 60% SW; $P < 0.02$, 75% SW; $P < 0.01$, 125% SW) (Fig. 6).

DISCUSSION

The response of blood ions to changes in external salinity shows that the chiton *Mopalia muscosa* does not regulate the Na^+ and Cl^- concentrations of its blood in either hyposmotic (60% SW) or hyperosmotic (125% SW) media. Although the K^+ and Mg^{2+} concentrations of the blood of 60% SW acclimated chitons are greater than the respective SW concentrations, these differences are small.

The blood Ca^{2+} concentration of hyposmotically stressed chitons is hyperregulated. Regulation of blood Ca^{2+} concentration has been shown in other hyposmotically stressed molluscs, such as the limpet *Acmaea* (= *Notoacmea*)

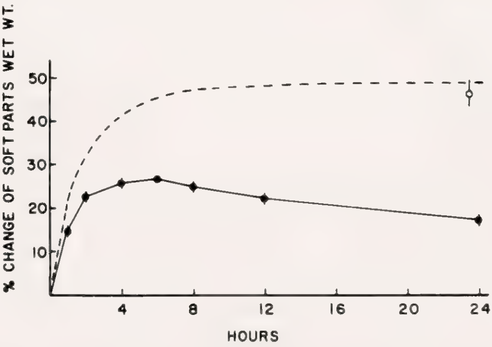


FIGURE 5. Comparison of the rate of weight change of fall *M. muscosa* responding, theoretically, as osmometers (dashed line) with the observed rate of weight change (solid circles), N = 6, vertical bars = ± 1 SE. All data are based on wet weight of the soft parts. Predicted steady-state weight change of chitons responding as if they were osmometers (open circle).

scutum from estuarine environments (Webber and Dehnel, 1968) and the chiton *Nuttalina californica* (Piper, 1975). The significance of hyper-regulation of blood Ca^{2+} concentration in molluscs is not known, but it could be of possible physiological benefit to the organism. Extracellular Ca^{2+} is involved in release of neurotransmitters from presynaptic neurons of the squid *Loligo vulgaris* (Miledi, 1973), and the action potential in the heart of the bivalve *Modiolus demissus* is dependent primarily on Ca^{2+} (Wilkens, 1972). Furthermore, ciliary movement is dependent on extracellular Ca^{2+} concentration (Eckert, 1972; Murakami and Eckert, 1972).

The blood Na^+ and Cl^- concentrations are reduced most rapidly during the first 2 hr of exposure to 60% SW (Fig. 1). The reduction of blood salt concentration could be due to salt loss (either diffusive across the body wall or through the urine) and to dilution of the blood by the water influx. The relative importance of salt loss and water influx in reducing blood Na^+ and Cl^- concentrations remains uncertain but cannot be ignored, since the diffusive salt loss would contribute to volume control by reducing the driving force for osmotic water flux existing across the body wall of hyposmotically stressed chitons. Diffusive salt loss in chitons has been demonstrated indirectly. Specimens of *Sypharochiton pelliserpentis* exposed to a seawater/sucrose mixture isosmotic to SW lost weight. This suggests that an isosmotic solution of salt and water was lost from the chitons, and that diffusive salt loss may contribute to volume control (Boyle, 1969).

Although blood osmotic pressure was not measured, the blood ion data, especially that of blood Na^+ and Cl^- concentrations, of salinity stressed chitons suggest that *M. muscosa* is an osmoconformer. Osmoconformity has been demonstrated in members of the genus *Mopalia* (Boyle, 1969); and the chitons *Cyanoplax hartwegii* (McGill, 1975), *Nuttalina californica* (Simonsen, 1975), and *Sypharochiton pelliserpentis* (Boyle, 1969) are osmoconformers in hypo- and hyperosmotic SW.

Since chitons are probably osmoconformers, intracellular volume regulation must occur during salinity stress. In most marine and estuarine invertebrates intracellular free amino acids are the source of solute for this regulation (Pierce

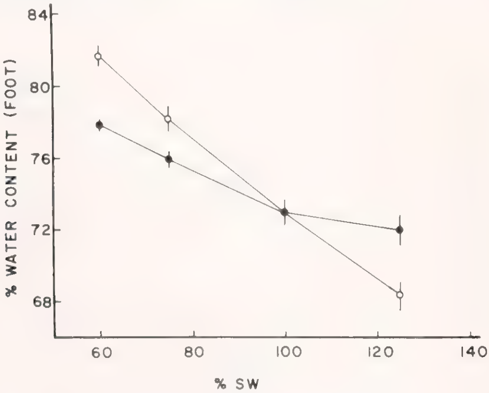


FIGURE 6. Water content of the foot muscle of *M. muscosa* as a function of salinity. Observed water content of muscle (closed circles); expected water content of muscle responding as if it were an osmometer (open circles). For chitons in 60, 75, and 100% SW, N = 10; 125% SW, N = 9. Vertical bars = ± 1 SE.

and Greenberg, 1973). Thus, osmotically stressed muscle tissue which relies on intracellular volume regulation would not be expected to alter its water content as would a Boyle-van't Hoff osmometer. This was found for the foot muscle-tissue of *M. muscosa* (Fig. 6).

Volume regulation has been studied in other chitons and in some bivalves. For example, the chiton *Cyanoplax hartwegii* can regulate volume in both 75 and 125% SW although the volume response is faster and more complete in 75% SW than in the hyperosmotic medium (McGill, 1975). McGill considers the hyposmotic volume-control response adaptive in chitons because it will limit swelling of the foot and allow continued attachment to the substrate. Volume regulation has also been studied in vertically separated populations of the chiton *Nuttalina californica.* Low-intertidal *Nuttalina* gain more weight in 50% SW than do high-intertidal specimens. Volume regulation was not demonstrated in either group; however, the maximum weight increase of both populations was less than that of chitons responding as if they were perfect osmometers (Simonsen, 1975). Likewise, the chiton *Sypharochiton pelliserpentis* does not regulate volume in hyposmotic SW. Instead, adaptation to hyposomotic media is accomplished by diffusive salt loss and by considerable tolerance to dilution of body fluids (Boyle, 1969). Volume regulation in hyposmotic SW has also been demonstrated in the bivalves *Modiolus demissus granosissimus* and *M. squamosus.* Volume control was not shown in either species when they were exposed to hyperosmotic SW (Pierce, 1971).

After a 24 hr exposure to either 60 or 75% SW (fall experiments), the chitons were returned to 100% SW, where they exhibited an undershoot of their original weight; that is, on return to 100% SW the chitons lost weight until they weighed less than their original weight at time zero. This may be due to loss of solute from the organism during exposure to hyposmotic SW (Gross, 1954). However, if this were the only cause the extent of undershoot should be correlated with salinity. This correlation was not found, suggesting that the chitons exposed to 60 and 75% SW lost the same amount of solute.

A somewhat surprising result of returning the chitons to 100% SW after exposure to hyposmotic SW was their steady weight gain after peak weight loss (Fig. 2). These chitons appear to begin volume regulation within 6 hr on return to 100% SW, whereas chitons exposed to 125% SW shrink passively and show limited ability to regulate volume. Thus, previous exposure to hyposmotic SW appears to activate volume regulation of *M. muscosa* re-exposed to 100% SW. These results differ from those of Pierce (1971) in a study of volume regulation in various species of *Modiolus.* Re-exposure of *Modiolus demissus granosissimum, M. modiolus, M. demissus,* and *M. squamosus* to full-strength SW after exposure to hyposmotic SW resulted in an undershoot of their original weight, and none of the species examined was able to regain its original volume. The physiological basis for this difference between chitons and bivalves is not known.

Volume control in an organism exposed to hyposmotic media may begin before the usually observed decline in weight. For example, *M. muscosa* begins to limit its rate of weight increase after 1 hr of exposure to hyposmotic SW (Fig. 5). Therefore, volume control mechanisms in *Mopalia* begin to function several hours before the observed decline in weight. Likewise, Machin (1975), using a graphic analysis of weight and water-content regulation, demonstrated that volume control in the polychaete *Glycera dibranchiata* begins before osmotic equilibrium is reached. The volume control mechanisms responsible for the chitons' early deviation from perfect osmometer behavior are not known. However, this effect could result from a change in epithelial permeability to water, an increase in water excretion or diffusive salt loss.

Low temperature (7° C) reduced rate of weight gain of *M. muscosa* exposed to 60% SW. Likewise, the weight-loss rate was reduced during volume regulation. Therefore, temperature seems to affect processes controlling both osmotic influx and volume regulation.

Volume regulation in *Mopalia* may vary with season (Fig. 3). Chitons on the U. S.-Canadian West Coast reproduce in the spring, and this difference could be related to the animals' reproductive state. Furthermore, all chitons in this study were taken within a restricted vertical range in the intertidal zone: Animals separated vertically in the intertidal zone have different periods of exposure, and, as mentioned above, Simonsen (1975) has demonstrated that low-intertidal specimens of *Nuttalina californica* gain more weight in hyposmotic SW than high-intertidal animals. Thus, season and vertical position in the intertidal zone, should be considered in future studies concerning water balance of intertidal organisms.

In conclusion, *M. muscosa* responds to short-term salinity stress by quickly activating mechanisms which control volume. This is of adaptive significance to an organism inhabiting the intertidal zone, where salinity may change rapidly.

The results reported in this paper were part of a Master's thesis submitted to the Graduate School of the California State University at Hayward. The authors wish to thank Dr. John Martin of the Moss Landing Marine Laboratories for allowing us to use the atomic absorption spectrophotometer. We also thank Dr. Robin Burnett of Hopkins Marine Station of Stanford University for checking some of the calculations in this paper. We thank Dr. Ralph Smith of the University of California at Berkeley, and Dr. Sidney Pierce, Jr., and Chuck Costa, both of the University of Maryland at College Park, for critically reviewing the manuscript.

SUMMARY

1. The effects of external salinity changes on whole-animal volume, blood ions, and muscle-tissue water content of the chiton *Mopalia muscosa* were investigated. The data indicated that short-term low-salinity adaptation in the chiton *M. muscosa* is achieved by volume control mechanisms that are quickly activated.

2. Blood Na^+, Cl^-, K^+, and Mg^{2+} were isoionic to the SW concentration in salinities ranging between 60 and 125% SW. Blood Ca^{2+} concentration was hyperregulated in hyposmotic SW.

3. Regulation of cell volume occurred in salinity-stressed chitons, as water content of foot-muscle tissue was regulated in both hypo- and hyperosmotic media.

4. When exposed to hyposmotic SW the chitons at first (0–4 hr) gained weight; this was followed by a period in which the rate of weight gain approached zero (4–6 hr) and finally by a period of weight loss (6–24 hr) (volume regulation). Exposure to hyperosmotic SW resulted in weight loss and little volume regulation.

5. Volume control in hyposmotic media is accomplished, in part, by a loss of solute.

6. Low temperature, 7° C, decreased both the water influx and volume regulation.

7. Volume regulatory mechanisms are activated within 1 hr after exposure to 60% SW.

LITERATURE CITED

Boyle, P. R., 1969. The survival of osmotic stress by *Sypharochiton pelliserpentis* (Mollusca: Polyplacophora). *Biol. Bull.,* **136**: 154–166.

Eckert, R., 1972. Bioelectric control of ciliary activity. *Science,* **176**: 473–481.

Fletcher, C. R., 1974a. Volume regulation in Nereis diversicolor—I. The steady state. *Comp. Biochem. Physiol.,* **47A**: 1199–1214.

Fletcher, C. R., 1974b. Volume regulation in Nereis diversicolor—III. Adaptation to a reduced salinity. *Comp. Biochem. Physiol.,* **47A**: 1221–1234.

Gross, W. J., 1954. Osmotic responses in the sipunculid *Dendrostomum zostericolum. J. Exp. Biol.,* **31**: 402–423.

Lange, R., and A. Mostad, 1967. Cell volume regulation in osmotically adjusting marine animals. *J. Exp. Mar. Biol. Ecol.,* **1**: 209–219.

Machin, J., 1975. Osmotic responses of the blood worm *Glycera dibranchiata* Ehlers: A graphical approach to the analysis of weight regulation. *Comp. Biochem. Physiol.,* **52A**: 49–54.

McGill, V. L., 1975. Responses to osmotic stress in the chiton, *Cyanoplax hartwegii* (Molluscs: Polyplacophora). *Veliger,* **18** (Suppl.): 109–112.

Miledi, R., 1973. Transmitter release induced by injection of calcium ions into nerve terminals. *Proc. R. Soc. Lond. B. Biol.,* **183**: 421–425.

Murakami, A., and R. Eckert, 1972. Cilia: activation coupled to mechanical stimulation by calcium influx. *Science,* **24**: 1375–1377.

Oglesby, L. C., 1975. An analysis of water content regulation in selected worms. Pp. 181–204 in F. J. Vernberg, Ed., *Physiological Ecology of Estuarine Organisms.* University of South Carolina Press, Columbia, South Carolina.

Pierce, S. K., Jr., 1971. Volume regulation and valve movements by marine mussels. *Comp. Biochem. Physiol.,* **39A**: 103–117.

Pierce, S. K. Jr., and M. J. Greenberg, 1973. The initiation and control of free amino acid regulation of cell volume in salinity-stressed marine bivalves. *J. Exp. Biol.,* **59**: 435–440.

Piper, S. C., 1975. The effects of air exposure and external salinity change on the blood ionic composition of *Nuttalina californica. Veliger,* **18** (Suppl.): 103–108.

Schales, O., and S. S. Schales, 1941. A simple and accurate method for the determination of chloride in biological fluids. *J. Biol. Chem.,* **140**: 879–884.

Simonsen, M., 1975. Response to osmotic stress in vertically separated populations of an intertidal chiton, *Nuttalina californica. Veliger,* **18** (Suppl.): 113–116.

Sokal, R. R., and F. J. Rohlf, 1969. *Introduction to Biostatistics.* W. H. Freeman and Co., San Francisco, 368 pp.

Stickle, W. B., and R. Ahokas, 1975. The effects of tidal fluctuation of salinity on the hemolymph composition of several molluscs. *Comp. Biochem. Physiol.,* **50A**: 291–296.

Watts, J. A., and S. K. Pierce, Jr., 1978. A correlation between the activity of divalent cation activated adenosine triphosphatase in the cell membrane and low salinity tolerance of the ribbed mussel, *Modiolus demissus demissus. J. Exp. Zool.,* **204**: 49–56.

Webber, H. H., and P. A. Dehnel, 1968. Ion balance in the prosobranch gastropod, *Acmaea scutum. Comp. Biochem. Physiol.,* **25**: 49–64.

Wilkens, L. A., 1972. Electrophysiological studies on the heart of the bivalve mollusc, *Modiolus demissus.* II. Ionic basis of the action potential. *J. Exp. Biol.,* **56**: 293–310.

Reference: *Biol. Bull.*, **159**: 376–393. (October, 1980)

ELECTRICAL ACTIVITIES IN THE SUBTENTACULAR REGION OF THE ANTHOMEDUSAN *SPIROCODON SALTATRIX* (TILESIUS)

KOHZOH OHTSU

Ushimado Marine Laboratory, Okayama University, Kashino, Ushimado, Oku, Okayama, 701-43 Japan

Electrophysiological studies of hydrozoans have revealed various kinds of electrical pulses of epithelial, muscular, or nervous origin. One striking feature of hydrozoans is that the conducting systems of those pulses often lie parallel to one another. It is interesting to determine how such parallel systems function and therefore, much effort has been made to show the origin, nature, and function of each conducting system, *e.g.*, in *Hydra* (Passano and McCullough, 1962, 1964, 1965; Josephson and Macklin, 1967; Josephson, 1967; Macklin and Josephson, 1971; Rushforth and Burke, 1971; Rushforth, 1971), in *Tubularia* (Josephson and Mackie, 1965; Josephson, 1965, 1974; Josephson and Rushforth, 1973; Kruijf, 1977), in hydromedusans (Spencer, 1975, 1978; Mackie, 1975) and in siphonophores (Mackie, 1976a, 1978). *Spirocodon*, the hydrozoan medusa used in this study, also has such parallel systems in some tissues (Ohtsu and Yoshida, 1973). Only those in the area between the ocelli and the nerve ring called the "subtentacular region" will be considered here.

Spirocodon saltatrix has many morphological charactristics in common with other species of Anthomedusae (Horridge, 1955; Mackie and Passano, 1968; Spencer, 1979; King and Spencer, 1979); but it shows some striking differences from them in the marginal region of the umbrella.

Figure 1 shows a part of the subtentacular region. According to Uchida (1927), the subtentacular region is formed in the following manner: In early developmental stages, single ocelli are present at the base of the four perradial and the four interradial tentacles, close to the nerve rings. As tentacles grow, these ocelli move away from the inner margin of the umbrella, forming an intervening expanse of tentacular tissues, the radial streaks, between the ocelli and the nerve ring while new ocelli and tentacles are formed at both sides of and slightly below those already formed. The newer the ocelli, the shorter the radial streaks. Thus a crescent-like area called the subtentacular region is produced between the ocelli (tentacular bases) and the nerve rings (velar base). A fully grown medusa has eight subtentacular regions at the lower margin of the umbrella. In large specimens, each subtentacular region contains more than 60 radial streaks. Sketches of the subtentacular region and the whole body are shown in Ohtsu and Yoshida (1973).

The subtentacular region should provide pathways for excitations traveling from the periphery (tentacles and ocelli) to the center (nerve ring). Histological as well as electrophysiological investigations were carried out to identify cell types within the subtentacular region in order to provide a morphological basis for interpreting data on pulse generating sites characterizing each conducting system.

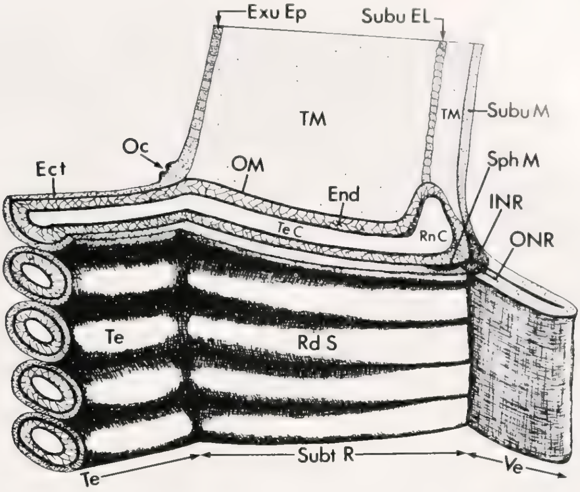


FIGURE 1. The subtentacular region. Four and a half radial streaks are shown. Ect, ectoderm; End, endoderm; Exu EP, exumbrellar epithelium; INR, inner nerve ring; TM, transparent mesogloea; Oc, ocellus; ONR, outer nerve-ring; Rd S, radial streak; Rn C, ring canal; Sph M, marginal sphincter muscle; Subt. R, subtentacular region; Subu EL, subumbrellar endodermal lamella; Subu M, subumbrellar muscle sheet; Te, tentacle; Te C, tentacular canal; OM, opaque mesogloea; Ve, velum. For explanation, see text.

MATERIALS AND METHODS

Specimens of *Spirocodon saltatrix* were collected from the Seto Inland Sea, Japan, and kept in running sea water. Half of the subtentacular region, with a small fraction of mesogloea lying under it, was dissected from the margin of the umbrella and used for histological observations and electrical recordings.

For electron- and light-microscopical observations, the ocelli and radial streaks were dissected from the subtentacular region. They were fixed in chilled 2% glutaraldehyde for 2 hr and post-fixed in 2% osmium tetroxide for 2.5 hr. To regulate osmolarity, 0.9 M sucrose or sea water was used. In some cases, fixation was done at 15°C for the first hour to retain microtuble structure. Fixatives were buffered with 0.1 M Na-cacodylate, pH 8.0. Post-fixation was omitted in the dye-marking experiment described later. The specimens were dehydrated through an ethanol series and embedded in TAAB embedding resin (TAAB Laboratories). Sections were cut with a Porter-Blum MT-2B ultramicrotome. Silver to gray sections were stained with uranyl acetate and then lead citrate and observed with a Hitachi electron microscope (HS-8). Thicker sections of 1–2 μm were stained with methylene blue for light microscopy.

Experiments were performed at room temperature (18–21°C). The arrangement of the recording chamber was similar to that described in Ohtsu and Yoshida (1973). During recording, the surface of the preparation was exposed to air to reduce short-circuit currents. During intracellular recording, preparations were almost totally immersed in sea water. Recording electrodes were advanced with a hydraulic micromanipulator in conjunction with a mechanical advance.

For extracellular recordings, two types of glass micropipette electrodes were used: rigid or floating. The latter consisted of a coiled piece of tungsten wire (30 μm diameter) joined with wax to the proximal opening of a micropipette electrode tip a few millimeters long. This type of electrode allowed vigorous movements of the preparation without electrical artifacts. Both types of electrodes were filled with sea water. External tip diameters ranged from 1 to 5 μm.

For intracellular recordings, micropipettes were filled with 2 M KCl and had DC-resistances of 20–100 MΩ. Rigid electrodes were used. Preparations were treated with a 0.3–0.5% pronase/sea-water solution for 4–8 min to facilitate penetration, aided by a jolting device (Tomita *et al.*, 1967).

A 1–2 hr immersion in pronase was used to dissociate the endodermal cells for examination with Normarski optics (Leitz, Orthoplan).

To measure depth of penetration of the recording electrode tips, a pair of electrodes was used, unless stated otherwise. Initially, both electrodes were placed on the surface of the radial streak and then one electrode was advanced through the tissues, leaving the other on the surface. Because smooth penetration was impossible, depths were estimated during withdrawal of the electrode, using a micromanipulator scale.

A micropipette filled with M/2 K-ferrocyanide instead of sea water was used to mark the recording sites. The pipettes with K-ferrocyanide were not used as recording electrodes because leakage of the electrolyte from the large pipette tips might have deleterious effects and because tissue penetration with such pipettes often resulted in failure of electrophoretic ejection of the fluid, probably because the pipette tips became plugged with tissue debris during penetration. After recording, the pipette with K-ferrocyanide was attached to the recording electrode tip and the electrolyte was expelled into tissues by inonophoretic current. One or two drops of ferric chloride solution were delivered to make a Berlin blue precipitate. Cross-sectional dimensions of tissues were estimated from light micrographs compared with readings from the micromanipulator scale.

The most serious difficulty with the rigid electrode was immobilizing the preparations. Since tissues can contract locally, mechanical restraint was impossible. Instead, various anaesthetics, such as urethane, menthol, MS-222, and magnesium chloride, were tried. Urethane was most successful. The urethane concentration was varied within the range of $1.0\text{–}3.0 \times 10^{-1}$ M, since the effect was not the same on every preparation.

Pulses can be evoked by termination of adapting light (Ohtsu and Yoshida, 1973). Light from a tungsten lamp (35 W) was delivered through a heat-absorbing filter and a light guide, the illuminance of which was 3500 lux in the plane of the preparation.

Electrical stimulation was by means of paired silver wires of 200 μm diameter through an isolation unit (Nihon Kohden, MSE-JM). In some cases, however, stimuli were delivered through the recording electrode.

Electrical responses were fed to a two-channel preamplifier formed by two FET-coupled operational amplifiers (Teldyne Filbrick, 142502) arranged in parallel. One was equipped with a Wheatstone bridge circuit, permitting measurement of membrane resistance changes. Two DC/AC amplifiers (Nihon Kohden, AVM-9S, AVH-9) were employed; the former, equipped with a beam chopper device, was most frequently used. The latter was used as a differential amplifier or to monitor photosignals. All responses were displayed on a dual-beam cathode ray oscilloscope (Nihon Kohden, VC-9A), photographed by a continuous recording camera (Nihon Kohden, PC-2B) and visually monitored on a second oscilloscope (Iwatsu, SS-5100).

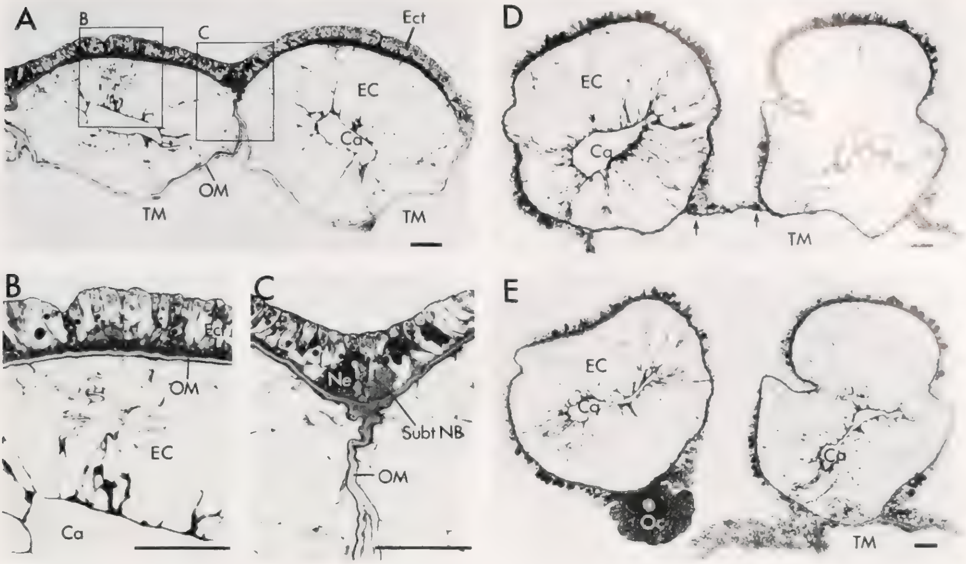


FIGURE 2. Photomicrographs of cross-sections through the radial streaks. Ca, canal; EC, endodermal cell; Ect, ectoderm; Ne, nematocyst; Oc, ocellus; OM, opaque mesogloea; Subt NB, subtentacular nerve bundle; TM, transparent mesogloea. Arrows in D show the subtentacular nerve bundles. Horizontal bars, 50 μm.

RESULTS

Histological observations

The ectoderm of the radial streaks is similar to that of the tentacles of other hydromedusans. Figure 2A shows a cross-section of two radial streaks. Figures 2B and C show enlargements of parts of A. Endodermal epithelial cells, surrounding the tentacular canal situated at the center, project their fine cytoplasmic processes towards a surrounding thin mesogloeal layer, resulting in many large endodermal spaces separated by the processes. The endodermal spaces may look larger in the photomicrographs than they actually are (Fig. 2) because the fine endodermal processes are often smaller than 0.1 μm, and thus beyond the limit of resolution of the light microscope. The mesogloeal layer is far more opaque than the jelly-like mesogloea found elsewhere. Therefore it will be called the opaque mesogloea and the latter, the transparent mesogloea. A layer of ectodermal cells surrounds the opaque mesogloea. The transparent mesogloea cannot be seen because it has the same refractive index as the embedding resin.

The subtentacular region is folded between adjacent radial streaks, forming a groove (Fig. 2A) where a single nerve bundle runs (Fig. 2C). This bundle will be called a subtentacular nerve bundle. The grooves become flatter near the nerve-rings. In contrast, going towards the ocelli away from the nerve-rings, the grooves become deeper, *i.e.*, the ectodermal cell layer extends progressively around the endoderm, while the subtentacular nerve bundle divides into two branches, each running in a corner of the groove (Fig. 2D). When the ectoderm completely surrounds the endoderm, the ocelli appear in the ectoderm on the exumbrellar side (Fig. 2E). Beyond the ocelli, the radial streaks become the tentacles.

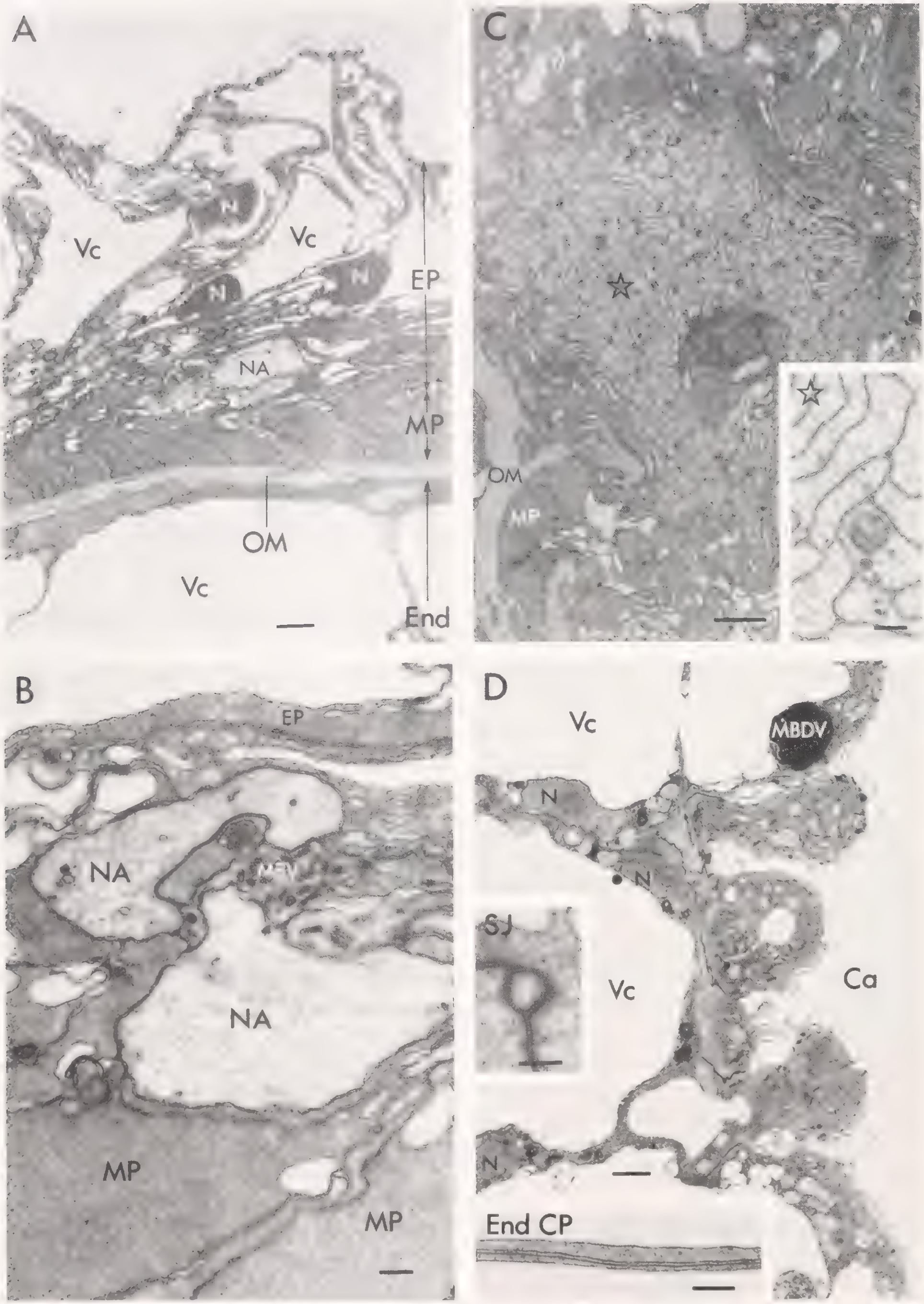

A
Vc
N
Vc
N
N
EP
NA
MP
OM
Vc
End
C
☆
OM
MP
☆
B
EP
NA
MBV
NA
MP
MP
D
Vc
MBDV
N
N
SJ
Vc
Ca
N
End CP

FIGURE 3. Electron micrographs of cross-sections through the radial streaks. A, the ectoderm and a part of the endoderm; B, high magnification of the endoderm; C, the subtentacular nerve bundle and high magnification of a part of it (asterisk); D, endodermal cells. Ca, canal; End, endoderm; End CP, fine endodermal cell process; EP, the epithelial process of the myoepithelial cell; MBV, membrane bound vesicle; MBDV, membrane bound digestive

The construction of the tentacles, therefore, necessarily resembles that of the radial streaks. In the vicinity of the ocelli, the nerve bundles are missing, probably because the nerves have dispersed. It seems that at least some axons, if not all of them, receive input from the ocelli and function in the photoresponse. The major functions of the subtentacular nerve bundle, however, are unknown.

Figures 3A-C show the ultrastructure of the ectoderm in cross sections. The thickness of the layer usually lies in the range 10–50 µm though it varies in different preparations and in different parts of the sections. The muscular processes of the myoepithelial cells are smooth muscle fibers running along the axis of the tentacle. They lie in a layer on the innermost side of the ectoderm, anchored to the mesogloea (A). They are connected with each other by structures resembling the gap junctions reported in other hydrozoan cells (Hand and Gobel, 1972). The muscle layer becomes thinner towards the nerve-ring and thicker towards the tentacle. The epithelial proccesses lying at the outermost surface and forming almost the whole surface of the ectoderm contain the nuclei and extremely large vacuoles lying in relatively undifferentiated cytoplasm (A). The opaque mesogloea is perforated in places by a tissue similar to that of the endodermal cell processes, allowing direct contact between the ectoderm and the endoderm, as reported in other anthomedusans (Boelsterli, 1977).

A number of nerve axons with variable diameters (Figs. 3A, B) and sometimes neuronal somata of about 5–10 µm in diameter are present. Both contain a number of microtubules and often membrane-bound vesicles. They are almost always adjacent to the myoepithelial cells. Some cells with a number of membrane-bound vesicles (recalling neurosecretory granules), are also observed (Fig. 3B). Synapse-like structures, however, have not been found so far between the cells identified, though they are frequently observed around the ocelli (Toh *et al.*, 1979).

The subtentacular nerve bundles consist of a large number of fine axons which contain many microtubules and membrane-bound vesicles (Fig. 3C). The number of axons differs from preparation to preparation but gradually decreases going towards the ocelli. No neuronal somata have so far been found along the bundle. They probably lie elsewhere, *e.g.*, adjacent to the ocelli or outside the nerve bundle. It must be noted that muscle processes are weakly developed near the subtentacular nerve bundle.

Cnidocytes are also present among the myoepithelial cells in the subtentacular region but they are far less abundant than in the tentacles.

The fine structure of the endodermal epithelial cells is shown in Figure 3D. A single type of endodermal cell can be identified in the subtentacular region except near the ring canal, where endodermal nerves can be seen as reported in *Stomotoca* (Mackie and Singla, 1975) and *Polyorchis* (Singla, 1978; Spencer, 1979). The endodermal cell cytoplasm expands on the side facing the tentacular canal, and the endoplasmic reticulum, Golgi apparatus, and mitochondria are in this region. A number of microvilli and cilia are present on the surface adjacent to the canal. Septate desmosomes are also observed connecting cells on their lumenal sides (Fig. 3D, inset). Membrane-bound digestive vacuoles are often observed, suggesting active phagocytosis. The nuclei lie towards the periphery. More peripherally, the cell processes become thin but often form enlargements including mitochondria and even nuclei on occasion, and extending in sheets of two

---

vacuole; MP, the myonal process of the myoepithelial cell; N, nucleus; NA, nerve axon; OM, opaque mesogloea; SJ, septate junction; Vc, vacuole. Horizontal bars, 0.2 µm in C and D insets and 2 µm in the others.

or more layers as far as the opaque mesogloea, creating many large spaces between the cell processes (Figs. 2, 3A, 3D inset). Where they reach the opaque mesogloea, the epithelial cell processes line its inner side (Fig. 3A).

Although the large endodermal spaces seen among the epithelial cell processes appear to be extracellular, this may not be the case. The epithelial process almost always consists of at least two layers of endoderm (Fig. 3D, inset). The narrow gap between the two layers is extracellular space, often observed to be connected with the central canal via a septate junction. If the large endodermal spaces were extracellular spaces, then they, too, should often be connected to the canal. But such cases have not been observed in electron micrographs. It is likely, therefore, that the large endodermal spaces represent extraordinarily large vacuoles in endodermal epithelial cells. Supporting evidence was obtained from experiments involving dissociation of the tissues with pronase. As the endodermal cells dissociate, the endodermal spaces become spherical and some of the cells break free as single spherical cells. Vacuoles occupy the greater part of the cytoplasm and the nuclei and major portion of the cytoplasm lie near the periphery of the cell. Cells isolated from the endoderm vary greatly in size. For instance, in one treatment the cells measured 15–20 μm and sometimes 30 μm, but in another treatment some cells measured 100 μm.

Conduction velocities of pulses recorded

When the recording electrode is placed at the surface of the subtentacular region, three types of pulses are recorded: slow monophasic pulses (SMPs; Figs. 4A, C), quick and slow compound pulses (QSCPs; Figs. 4A–D), and tentacle contraction pulses (TCPs; Fig. 4D). The first two were reported by Ohtsu and Yoshida (1973). The QSCP consists of a very short phase preceding a slow

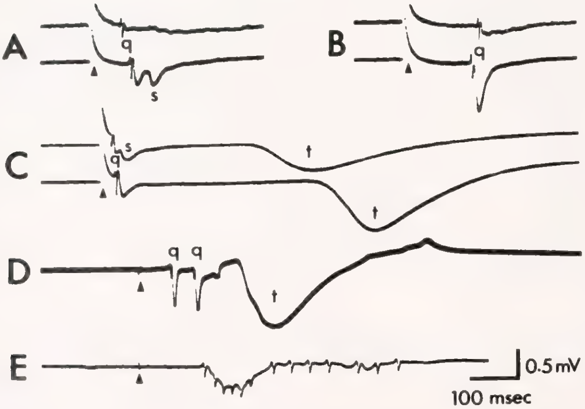


FIGURE 4. Pulses recorded in the subtentacular region. A and B show conduction of QSCPs. In A, a single electrical stimulus was delivered to a part of a tentacle distal to the recording sites. In B, a stimulus was delivered to an adjacent tentacle. C shows TCPs (t) following long after QSCPs and an SMP. D shows a TCP (t) following soon after two successive QSCPs, suggesting triggering of the TCP by the QSCPs. The distance between the recording electrodes and the stimulus site is much larger in D than C, as can be seen from the longer delay of the QSCPs after the electrical stimulus. E shows multiple firing of the quick phase of QSCPs over the course of the prolonged slow phase induced by urethane treatment. Arrows, electrical stimuli. "q" and "s" indicate a QSCP (or QSCPs) and an SMP, respectively.

phase which bears a remarkable resemblance to the SMP in terms of its time course and waveform (Fig. 4A).

A preliminary experiment showed that the conduction velocity of QSCPs in the subtentacular region is approximately 60 cm/sec (Ohtsu and Yoshida, 1973). This value represented only a single measurement, however, so further detailed bidirectional measurements were performed on the tentacles, taking advantage of their greater length. Electrical stimuli were employed to evoke QSCPs. At proximal parts of moderately relaxed tentacles, conductive velocities were calculated from the time difference between the quick components of the QSCPs recorded at two recording electrodes. For distal to proximal conduction, stimuli were delivered at distal parts of the tentacles with recording electrodes placed more proximally (Fig. 4A), while for proximal to distal conduction a neighboring tentacle was stimulated (Fig. 4B). In the latter case, QSCPs would first travel proximally up the stimulated tentacle and would reach the recording sites by travelling distally down the second tentacle after crossing the subtentacular region. The longer post-stimulus delays of the pulses in Figure 4B, compared with those in Figure 4A, reflect the longer distance travelled. The mean value obtained from 25 different tentacles from eight animals was 75 cm/sec (range, 50–104 cm/sec) in distal to proximal direction and 73 cm/sec (range, 57–95 cm/sec) in the opposite direction. In most cases, QSCPs appeared coupled with each other on a one-to-one basis within the subtentacular region and the tentacles associated with it.

SMPs can be conducted for only a short distance within the same radial streak (Ohtsu and Yoshida, 1973). The generating sites of spontaneously occurring SMPs frequently changed, as shown in Figure 7A, where with the first SMP pair, a pulse first appears on the lower trace and then on the upper trace, while in the second pair, pulses appear in the reverse order. Moreover, SMPs sometimes disappeared between the two electrodes and were not conducted (Fig. 4C). Although the pulses can be induced by termination of adapting light with latencies of 0.2–1.1 sec, probably mediated by the ocelli (Ohtsu and Yoshida, 1973), it was difficult to restrict pulse generation to a single fixed site; one should be cautious in estimating the conduction velocity because the pulses were often generated between two recording electrodes. Waveforms of SMPs became variable as they moved distally along the tentacles so that it was often difficult to identify the corresponding phases of pulses recorded at two points. Estimation of conduction velocity, therefore, was performed in the subtentacular region, using spontaneous pulses or pulses triggered by lights-off and measuring the peak-to-peak time delay, since the initial rising phase was not steep enough to allow initiation time to be accurately determined. Cases with extremely short time delays were excluded, as they were probably due to initiation of pulses between the two recording electrodes. The mean value from 32 different radial streaks from eight animals was 16 cm/sec (range, 9–38 cm/sec). It must be noted that SMPs with conduction velocities less than 9 cm/sec were seen, especially during sequences where later pulses tended to be conducted far more slowly than earlier ones. However, these data were omitted from the above measurements, since they were obtained in experiments using urethane. There may be more than one conducting pathway for SMPs.

When electrical recordings were performed on the subtentacular region or the tentacles, extremely slow deflections with monophasic and sometimes more complicated waveforms were observed, accompanied by muscle contractions (Fig. 4C); the waveforms may include electrical artifacts due to tentacular movements. These pulses are referred to hereafter as tentacle contraction pulses (TCPs).

Measurement of their conduction velocity was done as for SMPs but using pulses elicited by electrical stimulation on the tentacles and recorded in the subtentacular region. Recordings were not made on the tentacles because the pulses were larger and of simpler waveform on the subtentacular region. The mean value from 29 different radial streaks from nine animals (range, 2.2–8.6 cm/sec), which is very slow compared with the conduction velocity of other pulses, suggests that TCPs are conducted without involvement of the nervous system. Figure 4C illustrates the great difference in the conduction velocities of QSCPs and TCPs, whereas the TCP in Figure 4D occured shortly after the QSCPs. A possible explanation for this is that TCPs can be induced directly by QSCPs in the course of conduction. Such examples, indicating triggering of TCPs, were often encountered when QSCPs appeared in rapid succession.

Waveform changes of pulses associated with electrode insertion depth

To determine where QSCPs originate, one of a pair of recording electrodes was inserted into a radial streak, while the other was placed on the surface. Urethane at 2×10^{-1} M concentration was used to immobilize the preparation although this concentration of urethane did not always completely abolish movement. This treatment, however, often prolonged the duration of the slow phase of QSCPs considerably and sometimes resulted in multiple firing of the quick phase over the course of the slow phase (Fig. 4E). This was never seen in untreated preparations.

Immobile preparations with normal pulse waveforms were selected for these experiments. Treatment with urethane further abolished the lights-off evocation of QSCPs and SMPs so that the pulses had to be evoked by electrical stimulation of distal parts of the tentacles, resulting in the disappearance of locally conducted SMPs.

During insertions of recording electrodes into the radial streak, smooth penetration was always interrupted in two places, once near the surface and again at considerable depth. The depths at which these interruptions occurred seem to correspond to the interfaces between the ectoderm and opaque mesogloea near the surface, and between the opaque and transparent mesogloea on the opposite side of the radial streak (see Fig. 2).

Results of one penetration experiment are shown in Figure 5: When both recording electrode tips were placed side by side on the surface of a radial streak, QSCPs with the same waveform appeared in the upper and the middle traces, with no deflection in the differential, lower trace (A). At a depth of 635 μm, a very small deflection with a waveform similar to that of the upper trace was observed (B). It must be noted, however, that in B and also in the following records, the pulses recorded on the surface were smaller than the one in A, presumably due to damage to cells near the surface by the electrode as it advanced and as the wider part of tapered electrode tip entered the surface tissues. Withdrawal of the electrode in 70 μm steps resulted in reversal of the polarity of the deflection in the middle trace (C). As the electrode was withdrawn further, positivity of the middle trace was gradually increased (D, E) and reached a maximum in or near the canal (F–H). With further withdrawal the slow deflection gradually became less positive (I–K) and eventually reversed its polarity again (L). Approaching still close to the surface, the negative slow deflection grew larger (M, N) and reached its maximum amplitude on the ectodermal surface (O). These facts clearly indicate that the surface of the radial

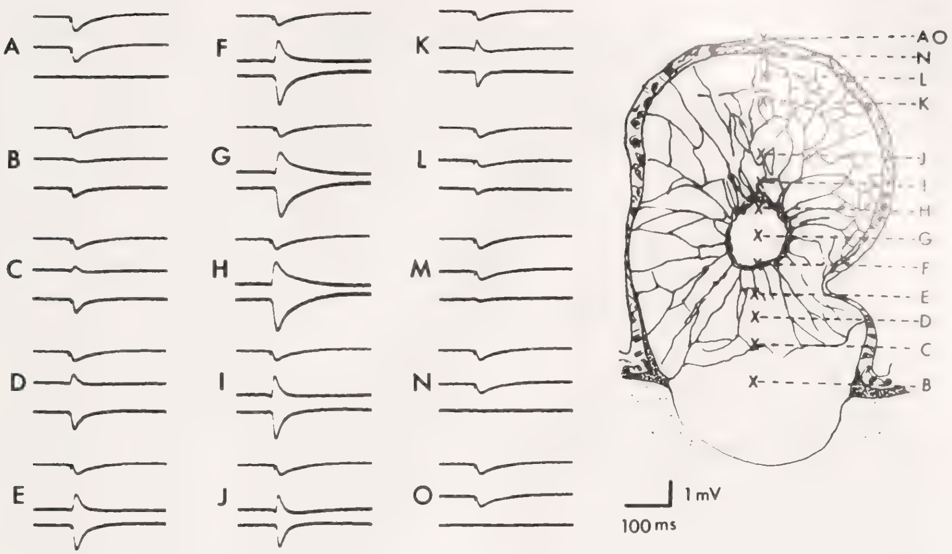


FIGURE 5. Waveform changes of QSCPs associated with the depth of the recording electrode tip. The recording electrode was inserted into the radial streak to a depth of 700 μm and then withdrawn stepwise. The upper traces were recorded from the surface electrode, the middle traces from the inserted electrode, and the lower traces show differential recordings (upper records minus middle records). The right inset diagram shows a cross-section of the radial streak used here. A, surface; B, 635 μm; C, 565 μm; D, 515 μm; E, 465 μm; F, 415 μm; G, 365 μm; H, 315 μm; I, 265 μm; J, 215 μm; K, 115 μm; L, 65 μm; M, 45 μm; N, 25 μm O, surface. QSCPs were evoked by electrical stimuli before initiation of the recordings.

streaks becomes negative (about 1.3 mV in H) compared with the central region of the endodermal canal during the slow phase of QSCPs. Similar results were obtained in four other preparations. Difference in DC-level measured between the ectoderm and the endoderm was variable, but in most cases was endodermal-side positive by a few millivolts.

The polarity of the quick phase of QSCPs, on the other hand, remained unchanged throughout the experiment. The amplitude was very small deep in the radial streak (Fig. 5B–J) and became larger when the electrode was moved to 100 μm or less from the surface (K–O). These changes are shown more clearly in Figure 6, where the quick phases were much larger than the slow phases, indicating that the recording site was nearer the nerve ring than the ocelli. The quick phases of QSCPs usually dominated the slow phase near the nerve ring, and vice versa near the ocelli; more nervous elements could be found near the former. Both the quick and slow phases of QSCPs abruptly increased when the electrode was withdrawn to within 40 μm of the surface of the ectoderm, strongly suggesting that both phases of QSCPs originate in the ectoderm. The waveforms of the pulses in Figure 6E closely resemble those at the surface (Fig. 6A) and such waveforms were often encountered when electrode tips obviously passed from the endoderm into the transparent mesogloea. This suggests that they are due to electrotonic spread from the surface. In the preparation, almost no deflection was observed in the endoderm, implying that the endodermal positive phase and the slow negative phase of QSCPs have different origins.

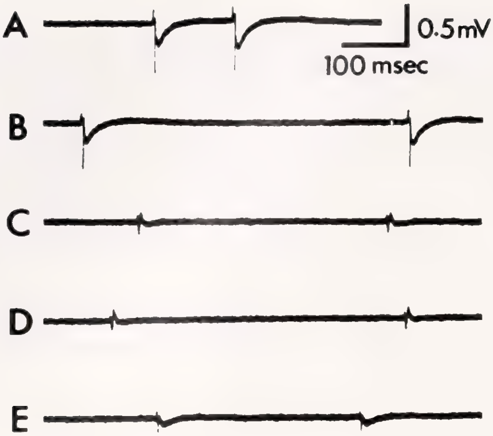


FIGURE 6. Amplitude changes of QSCPs associated with insertion depth of the recording electrode tip. Depth measurement was done with a single electrode. A, surface B, 40 μm; C, 90 μm; D, 190 μm; E, 290 μm.

Waveform changes of SMPs associated with electrode insertion depth

To permit SMPs to be triggered by lights-off stimulation, preparations were moderately anesthetized with 10^{-1} M urethane. Since this concentration did not immobilize animals completely, the experiment had to be done quickly. Initially, two electrode tips were placed a few mm apart from each other (not as in Figure 5, where they were placed directly side by side). The results are shown in Figure 7.

When both recording electrodes were placed on the surface of a radial streak, a pair of QSCPs and two pairs of SMPs appeared in both records (A). With the insertion of the electrode, very small negative deflections (B, upper trace) were seen. As the electrode was withdrawn the potentials reversed their polarity, increased in amplitude, and then became negative again, in a manner closely resembling Figure 5 (C–J). Similar results were obtained in 13 other experiments from seven animals. Positive pulses in the endodermal canal corresponded with QSCPs and SMPs and will be called PEPs. The maximum PEP was usually much larger than the maximum slow phase of QSCPs and the maximum SMP; in extreme cases without urethane treatment, the PEP reached about 4 mV, being four times larger than the corresponding SMP. The PEP sometimes showed a biphasic waveform with a negative phase, suggesting that it is a conducted event. It is noteworthy that QSCPs and SMPs together show coupling with PEPs, except for the last PEPs in D and E and the last SMP in the upper trace of J. These might have been lost in the course of conduction.

Pulses recorded form the ectoderm with high-resistance electrodes

When recording electrodes with resistances of 20–50 MΩ were placed on the surface of the radial streaks and brief vibrations were delivered by means of a jolting device (Tomita *et al.*, 1967), SMPs and the slow phase of QSCPs abruptly changed polarity immediately after a negative shift in the DC-level of 20–50 mV (mean, 37 mV; measurements were done in 12 cases). Large, positive, long duration pulses (ectodermal large pulses), amplitudes of which usually ranged between 5 and 10 mV, were common in the 26 cases studied. They always corresponded either to SMPs or to the slow phase of QSCPs (Fig. 8A). It is quite clear that they originate in certain ectodermal cells of the radial strands because the fine tips of electrodes used could not penetrate the opaque mesogloea without being damaged. To estimate membrane resistance changes during the large pulses, current pulses were delivered through a balanced bridge circuit but unexpectedly, no changes were observed in four cells measured (Fig. 8B). However, injection of positive current of about 10^{-8} A through the internal electrode often induced slow positive deflection similar to the ectodermal large pulses (Fig. 8D), resembling intracellularly recorded subthreshold pulses in the epithelio-muscular cell of *Nanomia* (Spencer, 1971). Pulses could not be evoked by delivering current extracellularly through such fine electrode tips. Even using electrodes with much larger tips of 20–30 μm, current of 10^{-5} A was needed. Furthermore, resistances were measured before and after withdrawal of the inserted electrode and a mean difference of 9.3 MΩ (variation, 3–4 MΩ) was obtained in five measurements. Therefore, it appears likely that the ectodermal large pulses are recorded within cells having an excitable membrane. Similarly, the experimental results indicate that SMPs and the slow phase of QSCPs originate in the same kind of cells and can therefore be regarded as extracellular forms of the ectodermal large pulses. The

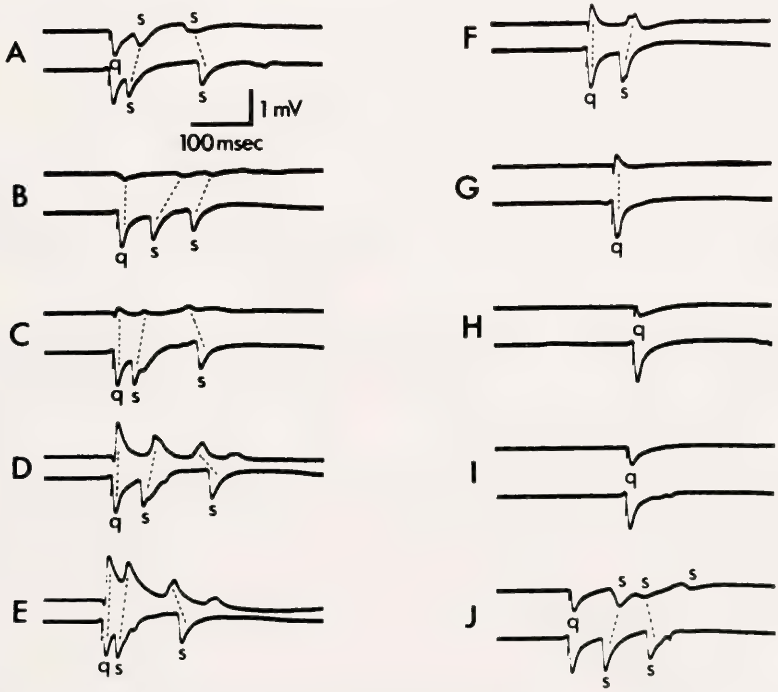


FIGURE 7. Waveform changes of SMPs (s) and QSCPs (q) associated with the depth of the recording electrode tip (upper trace). Lower trace is reference electrode on surface. A, surface; B, 460 μm; C, 360 μm; D, 310 μm; E, 210 μm; F, 110 μm; G, 60 μm; H, 40 μm; I, 20 μm J, surface. Originating sites of SMPs often changed; in the first pair of A they appeared first in the lower trace but in the second pair, the order was reversed. Dotted line shows possible pairs of pulses. All the pulses were evoked by terminating adapting light slightly before initiation of each recording.

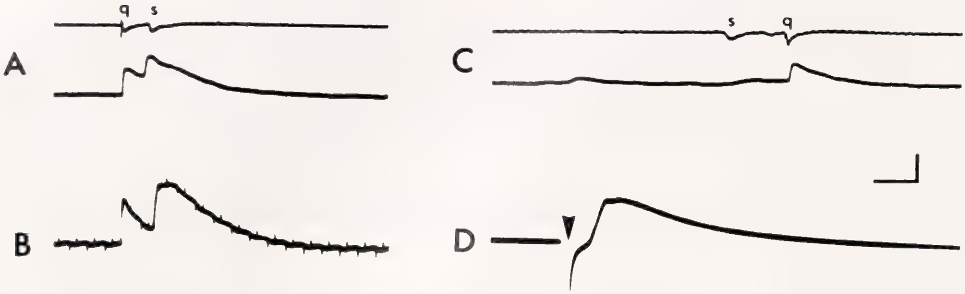


FIGURE 8. Intracellular recordings from ectoderm and endoderm. A: recording from the ectoderm of a radial streak with a high-resistance electrode (lower trace) and simultaneous extracellular recording from the ectodermal surface (upper trace), respectively. B: result of the resistance-change measurement. Current pulses with duration of 7 msec were delivered at 33 Hz. C: recordings from the endoderm of a radial streak with a high-resistance electrode (lower trace) and simultaneous extracellular recording from the ectodermal surface (upper trace). D: pulse evoked by electric current through a high-resistance electrode penetrating intracellularly in the ectodermal layer. Note the summation of ectodermal large pulses in A and B. Arrow: artifact due to current injection. Horizontal bar, 100 msec; vertical bar, 1 mV in the upper traces of A and C, 5 mV in B and the lower traces of A and C, 12.5 mV in D and 3.4 MΩ in B. q = QSCP, s = SMP.

two ectodermal large pulses occurring closely in time showed summation (Fig. 8A, B).

Pulses recorded from the endoderm with high-resistance electrodes

Since the fine tips of the high-resistance electrodes could not penetrate the opaque mesogloea, they were inserted into the endoderm of the radial streaks through cuts made at the bases of the tentacles. The insertion of electrodes with resistances of 80–100 MΩ sometimes resulted in positive pulses which were small, but somewhat larger than PEPs, showing amplitudes of 2–4 mV (Fig. 8C). Such pulses were obtained immediately after an abrupt negative shift of DC-level of 36–44 mV (mean, 40 mV in eight measurements). These pulses will be called endodermal large pulses. In Figure 8C, the SMP did not appear coupled with the endodermal large pulses because the inserted electrode was separated from the surface electrode by a distance greater than SMPs could travel, whereas in other records with shorter distances, SMPs and the endodermal large pulses often showed correspondence. Membrane resistance changes during the course of the endodermal large pulses could not be measured reliably because the high resistance of the electrodes resulted in unstable tip resistance. In one case where tip resistance was relatively stable, the resistance was measured before and after withdrawal of the inserted electrode and a difference of 13 MΩ was obtained, suggesting that the endodermal large pulses are also of intracellular origin.

DISCUSSION

QSCPs and SMPs rapidly increase in amplitude as the electrode is withdrawn to within 100 μm of the tissue surface and reach their maximum amplitudes at the ectodermal surface, suggesting that they are generated by ectodermal cells. A depth of 100 μm is greater than the thickness of the ectodermal layer (10–50 μm). The effects may be attributed to a current-guide effect of the hole made in the tissues (especially in the opaque mesogloea) by the shank of the electrode during deep penetration.

The quick phase and the slow phase of QSCPs appear to have their origins in different cells, because the quick phase can be isolated using urethane (Fig. 4E). On the other hand, the slow phase of QSCPs and SMPs becomes smaller towards the nerve ring while the quick phase of QSCPs gets larger. This matches the histological observation that in this region, the muscle process of the myoepithelial cells become weaker and more nervous elements are observed. The slow phase of QSCPs and SMPs appear coupled with PEPs, and SMPs cannot usually cross over the groove between two radial streaks, where few myonal processes can be observed. This suggests that the muscle processes may generate the slow phase of QSCPs and SMPs; and the nervous elements, the quick phase of QSCPs. The latter seems more likely given the fact that the quick components have similar conduction velocities to pulses in the outer nerve-ring (nMPs, Ohtsu and Yoshida, 1973) and are usually coupled to them on a one-to-one basis. Indeed, a number of axons are observed among the myoepithelial cells.

It is not clear if SMPs are triggered events or not. Speculating from the fact that SMPs occur at lights-off, they seem to be triggered by something that could be described as a light-information carrier system. SMPs can also originate spontaneously without light stimuli. Non-nervous elements do not generally show repeated spontaneous firings, whereas SMPs do. Furthermore, extremely small deflections with very short duration were sometimes observed in the subtentacular region (Fig. 9A). It is likely, therefore, that those pulses trigger SMPs. However, recordings are not sufficient to permit definite conclusions. Another possibility is that conduction of SMPs is linked with the conducting system of PEPs.

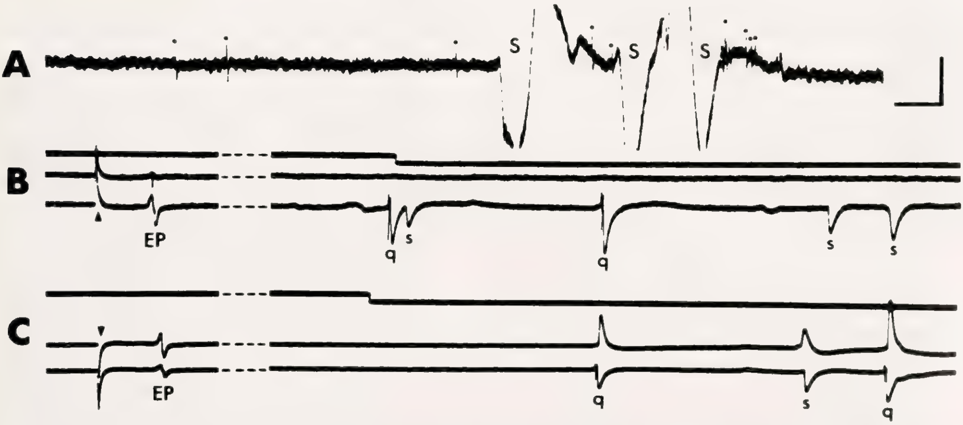


FIGURE 9. A: extremely small pulses (dots) recorded from the surface of a radial streak. B: simultaneous recordings from the exumbrellar epithelium near the ocelli (middle trace) and surface of a radial streak (lower trace). The first QSCP (q) and SMP (s) are spontaneous. Note: epithelial pulses (EPs) can invade into the radial streak, where they have longer durations and larger amplitudes than in the exumbrella, but QSCPs and SMPs are not conducted to the exumbrella. C: simultaneous recordings from the endoderm (middle trace) and the ectoderm (lower trace) of a radial streak. The PEPs (middle trace) appear coupled with the QSCPs and the SMPs. The EPs were evoked by a single shock of electrical stimuli (arrows) delivered to the exumbrella and the QSCPs and the SMPs were induced by lights-off (downward shifts of the upper traces in B and C). Horizontal bar, 100 msec; vertical bar, 0.1 mV in A, 0.4 mV in B and 1 mV in C.

Although intracellular dye-marking was not feasible, it appears from Figure 8 that the ectodermal large pulses are recorded intracellularly from the cells which also generate SMPs and the slow phase of QSCPs extracellularly. Histological investigations have revealed at least three types of cells—myoepithelial cells, nerve cells, and nematocytes—in the ectodermal layer of the subtentacular region. The latter two seem not to be responsible for generating the ectodermal large pulses because the region used for experiment includes only a small number of nematocytes, and neuronal somata large enough to be penetrated intracellularly are rare. Penetrations regularly showing the ectodermal large pulses would not be expected with so few cells. The epithelial processes of the myoepithelial cells, which cover almost all the subtentacular region, are therefore the most probable sites of origin of the ectodermal large pulses.

If the myoepithelial cell is penetrated by a micropipette electrode, then it is likely that the tip of the electrode usually lies in a large vacuole. This may explain why no resistance change is observed; the membrane of the vacuole, which might have a high resistance, impedes the resistance change occurring in the cytoplasmic membrane from reaching the inside of the vacuole.

Another possible reason for the lack of resistance changes is that some sensitive cells are actively excited while other, less sensitive ones receive passively conducted depolarizations spreading electronically from the former. The cell generating the ectodermal large pulses in Figure 8B might belong in the latter class, the loss of excitability being caused by the electrode impalement or some other factor. Electronic spread from other cells is possible if low-resistance intercellular pathways are present between the myoepithelial cells, as reported in other hydrozoan cells (Hand and Gobel, 1972; Mackie, 1976b; King and Spencer, 1979). Indeed, gap junctions are observed between muscle processes of *Spirocodon.*

When two pulses, whether QSCPs or SMPs, appear close together in time, summation is often observed in the ectodermal large pulses (Fig. 8A). This is reminiscent of the observation that closely spaced large QSCPs can evoke a TCP (Fig. 4D). The ectodermal large pulses, therefore, may develop into much larger action potentials inducing tentacular contraction when the membrane potential of the cell, probably the myoepithelial cell, is raised to a certain level (a critical depolarization potential) due to summation. This can be seen in the dissociated ectodermal epithelial cells of *Hydra,* in which spontaneous oscillations develop into large spikes (Kass-Simon and Diesl, 1977), and in the epitheliomuscular cell of the siphonophore *Nanomia,* where subthreshold pulses are thought to develop electrical responses with larger amplitude, causing twitch responses (Mackie, 1976a).

The mechanism generating PEPs is puzzling. The reversed polarity of PEPs and ectodermal slow pulses (Figs. 5, 7) suggests that the two are in a sink-and-source relationship in an electrogenerative system. Arguing against this idea is the long distance between the ectoderm, where SMPs (and the slow phase of QSCPs) are observed, and the central region of the endoderm, where the maximum PEPs are observed. Moreover, the opaque mesogloea between the ectoderm and the endoderm may function as a current barrier. The opaque mesogloea, however, is perforated in places with a tissue resembling an endodermal cell process, permitting endodermal cells direct contact with ectodermal cells. It is possible, therefore, that depolarizations of ectodermal cells reach electronically peripheral processes of endodermal cells and then are conducted electrogenerativly to the central region, generating PEPs.

The endodermal large pulses appear from Figure 8C to be recorded intracellularly from endodermal cells which also generate PEPs extracellularly. The small amplitude of the endodermal large pulses, however, argues against regarding them as intracellular recordings. The small amplitude is explicable if it is assumed (a) that the pulses recorded are due not to active excitation of the endodermal cells penetrated, but to electronic spread from other actively excited endodermal cells or (b) that recordings are from vacuoles.

PEPs sometimes show diphasic waveforms followed by negative deflections, suggesting that endodermal cells are excitable and conductive. If so, PEPs may be conducted back to ectodermal cells and cause small deflections. A similar case of endodermal pulses evoking pulses in ectodermal cells electrotonically has been reported in the siphonophore, *Nanomia* (Mackie, 1976a).

The pulses of *Spirocodon* are comparable to electrical potentials described in several hydrozoan medusae (*e.g.*, Mackie and Passano, 1968; Spencer, 1975; Mackie, 1975, 1976a; Spencer, 1978). As described above, the quick phase of QSCPs is best interpreted as a nervous event associated directly or indirectly with the outer nerve-ring, and the slow phase as an electrical concomitant of myoepithelial cell activity, triggered by the quick phase. In *Spiricodon* QSCPs (and SMPs) occurring singly are not usually accompanied by tentacular contractions, as far as can be observed under a dissecting microscope. This is different from *Proboscidactyla* (Spencer, 1975) but similar to *Sarsia* (Passano *et al.*, 1967). Tentacular contraction can be induced, however, when two pulses are close together in time, probably by summation or facilitation of two slow phases.

The slow phase of QSCPs decreases or disappears in excess Mg^{2+} and becomes larger stepwise by successive electrical stimuli, especially in a relatively depressed condition. In this respect, QSCPs obviously resemble the tentacle pulse (TP) in *Stomotoca* described by Mackie (1975); the quick phase appears to correspond to the pretentacle pulse (PTP) of Mackie (1975). Spencer (1978) has reported the TP without initial quick phase in the tentacles of *Polyorchis*. Also in *Spirocodon,* the quick phase was often extremely small, especially in tentacles, but QSCPs were readily distinguishable from SMPs and TCPs because the former was conducted only locally and the latter showed extremely slow conduction associated with muscle contraction.

Large specimens of *Spirocodon* with umbrellar diameter of 4–7 cm, as used in this study, show almost no crumpling in response to pricking the exumbrellar ectoderm with fine forceps. But such stimulation can evoke epithelial pulses (Ohtsu and Yoshida, 1973) similar to the CrPs of Spencer (1975, 1978). Small specimens of about 1 cm clearly show the crumpling response. Epithelial pulses distinct from SMPs and QSCPs (Fig. 9B, C) can also invade the subtentacular region, as in *Sarsia*. The pulses are larger in the endoderm than the ectoderm (Fig. 9C) but their generating origin remains to be investigated.

I should like to thank Professor G. O. Mackie of University of Victoria, Canada, and Dr. A. N. Spencer of University of Alberta, Canada, for their help in preparing this manuscript. Work supported in part by grants-in-aid to M. Yoshida from the Ministry of Education.

SUMMARY

1. Three types of cells—myoepithelial cells, nerve cells, and nematocytes—are found in the ectoderm of the subtentacular region except near the nerve ring. A

single type of endodermal cell is identified in the endoderm of the subtentacular region except near the ring canal.

2. Three types of conductive pulses—QSCPs, SMPs, and TCPs—are recorded in the ectoderm. When recording electrodes are inserted into the endoderm, SMPs and the slow phase of QSCPs (negative pulses) are replaced by PEPs (positive pulses).

3. Using high-resistance electrodes, two types of pulses (ectodermal and endodermal large pulses) are recorded (presumably intracellularly) from the ectoderm and the endoderm, respectively. They probably originate in the myoepithelial cells and in the endodermal cells, respectively.

4. SMPs and the slow phase of QSCPs occur coupled with the ectodermal large pulses and appear to be generated in the same cells, as the extracellular forms of the latter. The quick phase of QSCPs is probably an event generated by nerves in the ectoderm.

5. PEPs occur coupled with the endodermal large pulses and may be generated in endodermal cells as the extracellular forms of the endodermal large pulses.

6. Pulses with extremely small amplitudes suggesting the presence of another conducting system can be recorded.

7. SMPs and QSCPs are different from the crumpling pulses of Spencer (1975).

LITERATURE CITED

BOELSTERLI, U., 1977. An electron microscopic study of early developmental stages, myogenesis, oogenesis and cnidogenesis in the anthomedusa, *Podocoryne carnea* M. Sars. *J. Morphol.*, **154**: 259–290.

HAND, A. R., AND S. GOBEL, 1972. The structural organization of the septate and gap junctions of *Hydra*. *J. Cell Biol.*, **52**: 397–408.

HORRIDGE, G. A., 1955. The nerves and muscles of Medusae. II. *Geryonia proboscidalis* Eschscholtz. *J. Exp. Biol.*, **32**: 555–568.

JOSEPHSON, R. K., 1965. Three parallel conducting systems in the stalk of a hydroid. *J. Exp. Biol.*, **42**: 139–152.

JOSEPHSON, R. K., 1967. Conduction and contraction in the column of *Hydra*. *J. Exp. Biol.*, **47**: 179–190.

JOSEPHSON, R. K., 1974. Factors affecting muscle activation in the hydroid *Tubularia*. *Biol. Bull.*, **147**: 594–607.

JOSEPHSON, R. K., AND M. MACKLIN, 1967. Transepithelial potentials in hydra. *Science*, **156**: 1629–1631.

JOSEPHSON, R. K., AND G. O. MACKIE, 1965. Multiple pacemakers and the behaviour of the hydroid *Tubularia*. *J. Exp. Biol.*, **43**: 293–332.

JOSEPHSON, R. K., AND N. B. RUSHFORTH, 1973. The time course of pacemaker inhibition in the hydroid *Tubularia*. *J. Exp. Biol.*, **59**: 305–314.

KASS-SIMON, G., AND V. K. DIESL, 1977. Spontaneous and evoked potentials from dissociated epithelial cells of *Hydra*. *Nature*, **265**: 75–77.

KING, M. G., AND A. N. SPENCER, 1979. Gap and septate junctions in the excitable endoderm of *Polyorchis penicillatus* (Hydrozoa, Anthomedusae). *J. Cell Sci.*, **36**: 391–400.

KRUIJF, H. A. M., 1977. Bursting pacemaker activity in the solitary hydroid *Tubularia solitaria*. *J. Exp. Biol.*, **68**: 19–34.

MACKIE, G. O., 1975. Neurobiology of *Stomotoca*. II. Pacemakers and conduction pathways. *J. Neurobiol.*, **6**: 357–378.

MACKIE, G. O., 1976a. The control of fast and slow muscle contractions in the siphonophore stem. Pp. 647–659 in G. O. Mackie, Ed., *Coelenterate ecology and behavior*. Plenum Press, New York.

MACKIE, G. O., 1976b. Propagated spikes and secretion in a coelenterate glandular epithelium. *J. Gen. Physiol.*, **68**: 313–325.

MACKIE, G. O., 1978. Coordination in physonectid siphonophores. *Mar. Behav. Physiol.*, **5**: 325–346.

Mackie, G. O., and L. M. Passano, 1968. Epithelial conduction in Hydromedusae. *J. Gen. Physiol.*, **52**: 600–621.

Mackie, G. O., and C. L. Singla, 1975. Neurobiology of *Stomotoca*. I. Action systems. *J. Neurobiol.*, **6**: 339–356.

Macklin, M., and R. K. Josephson, 1971. The ionic requirements of transepithelial potentials in *Hydra*. *Biol. Bull.*, **141**: 299–318.

Ohtsu, K., and M. Yoshida, 1973. Electrical activities of the anthomedusan, *Spirocodon saltatrix* (Tilesius). *Biol. Bull.*, **145**: 532–547.

Passano, L. M., and C. B. McCullough, 1962. The light response and the rhythmic potentials of hydra. *Proc. Nat. Acad. Sci. U. S. A.*, **48**: 1376–1382.

Passano, L. M., and C. B. McCullough, 1964. Co-ordinating systems and behaviour in *Hydra*. I. Pacemaker system of the periodic contractions. *J. Exp. Biol.*, **41**: 643–664.

Passano, L. M., and C. B. McCullough, 1965. Co-ordinating systems and behaviour in *Hydra*. II. The rhythmic potential system. *J. Exp. Biol.*, **42**: 205–231.

Passano, L. M., G. O. Mackie, and M. Pavans de Ceccatty, 1967. Physiologie du comportement de l'hydroméduse *Sarsia tubulosa* Sars. Les systèmes des activités spontanées. *C. R. Acad. Sci. (Paris)*, **264**: 614–617.

Rushforth, N. B., 1971. Behavioral and electrophysiological studies of hydra. I. Analysis of contraction pulse patterns. *Biol. Bull.*, **140**: 255–273.

Rushforth, N. B., and D. S. Burke, 1971. Behavioral and electrophysiological studies of hydra. II. Pacemaker activity of isolated tentacles. *Biol. Bull.*, **140**: 502–519.

Singla, C. L., 1978. Fine structure of the neuromuscular system of *Polyorchis penicillatus* (Hydomedusae, Cnidaria). *Cell Tissue Res.*, **193**: 163–174.

Spencer, A. N., 1971. Myoid conduction in the siphonophore *Nanomia bijuga*. *Nature*, **233**: 490–491.

Spencer, A. N., 1975. Behavior and electrical activity in the hydrozoan *Proboscidactyla flavicirrata* (Brandt). II. The medusa. *Biol. Bull.*, **149**: 236–250.

Spencer, A. N., 1978. Neurobiology of *Polyorchis*. I. Function of effector systems. *J. Neurobiol.*, **9**: 143–157.

Spencer, A. N., 1979. Neurobiology of *Polyorchis*. II. Structure of effector systems. *J. Neurobiol.*, **10**: 95–117.

Toh, Y., M. Yoshida, and H. Tateda, 1979. Fine structure of the ocellus of the hydromedusan, *Spirocodon saltatrix*. I. Receptor cells. *J. Ultrastruct. Res.*, **68**: 341–352.

Tomita, T., A. Kaneko, M. Murakami, and E. L. Pautler, 1967. Spectral response curves of single cones in the carp. *Vision Res.*, **7**: 519–531.

Uchida, T., 1927. Studies on Japanese Hydromedusae. I. Anthomedusae. *J. Fac. Sci. Imp. Univ. Tokyo, Section IV*, **1**: 145–241.

Reference: *Biol. Bull.,* **159**: 394–401. (October, 1980)

EFFECT OF SYMBIOTIC ZOOXANTHELLAE AND TEMPERATURE ON BUDDING AND STROBILATION IN *CASSIOPEIA ANDROMEDA* (ESCHSCHOLZ)

M. RAHAT AND ORIT ADAR*

Department of Zoology, The Hebrew University, Jerusalem, Israel

Many algal-invertebrate symbioses are based on mutual biotrophic benefit. The heterotrophic host is supplied with photosynthates from its phototrophic symbiont, and the latter obtains both nutrients and an optimal habitat (Smith *et al.*, 1969; Cook, 1972; Richmond and Smith, 1979).

Symbiotic associations were probably initiated by a chance meeting of the cosymbionts. A loose faculative symbiosis could evolve in time into a symbiosis obligatory for at least one of the partners. Such symbioses can affect both form and function of the cosymbionts, and through coevolution new species may have evolved (Margulis, 1976). The symbiogenic theory of the origin of mitochondria and chloroplasts is based on the above assumptions.

To illustrate such evolutionary trends, the following examples are usually cited: The platyhelminth *Amphiscolops langerhansi* is unable to achieve sexual maturity without its symbiotic algae (Taylor, 1971), and the closely related flatworm *Convoluta roscoffensis* is completely dependent on its symbiotic algae "for its bulk nutrients and growth stimuli" (Provasoli *et al.*, 1968). It has also been claimed that the scyphozoan medusa *Mastigias papua* will strobilate ephyrae only if infected with zooxanthellae, and "if a scyphistoma is deprived of its zooxanthellae, it can never strobilate" (Sugiura, 1964). Ludwig (1969) stated similarily that aposymbiotic scyphistomae of *Cassiopeia andromeda* obtained in his laboratory "have only a limited vitality, and cease their strobilation completely".

The above statements allege an obligate dependence of the hosts on their symbiotic algae. It is thus of interest that all the above invetebrates form aposymbiotic eggs and larvae, and the symbiotic algae are acquired anew at each generation.

In view of this, we reexamined the life cycle of *C. andromeda.* In this study we report strobilation and formation of aposymbiotic ephyrae by aposymbiotic scyphistomae, and the effect of temperature on budding and strobilation.

MATERIALS AND METHODS

Mature *Cassiopeia* medusae were collected from the Gulf of Eilath, brought to the laboratory within 8 hr, and maintained in aquaria at 18° ± 2°C. During transport and in the aquaria, the medusae released many aposymbiotic planulae. Planulae left in the aquaria, or maintained in water from the latter, developed into polyps hosting algae (Fig. 1a). In addition to the polyps obtained from medusae collected at Eilath, polyps were also obtained from a culture maintained in our department for several years.

---

* This study is part of a Master's thesis submitted by the junior author to the Hebrew University.

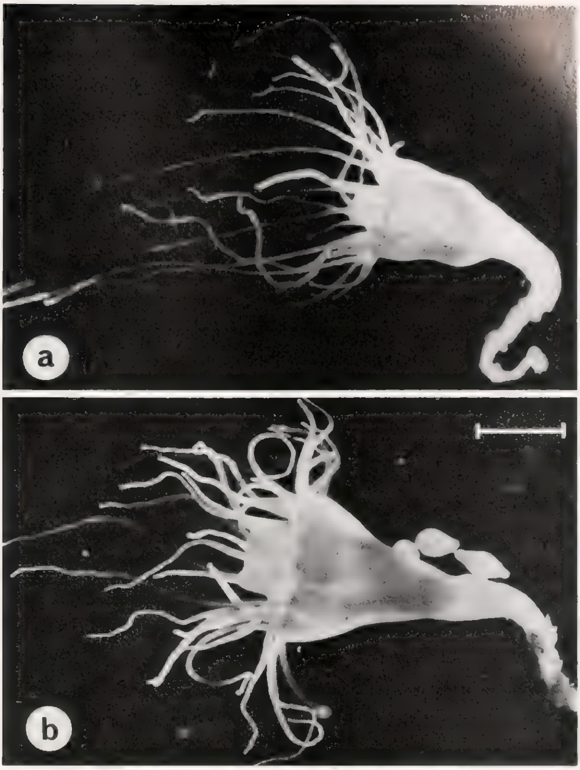


FIGURE 1. Polyps of *C. andromeda.* a. Symbiotic polyp. The dark dots in the calyx are endocellular zooxanthellae. b. Aposymbiotic polyp with three buds. Scale bar: 1 mm.

To obtain aposymbiotic polyps (Fig. 1b), planulae were collected from the aquaria, and washed and maintained in sterile sea water, in crystallizing dishes or finger bowls.

Aposymbiotic polyps were also obtained by incubation of symbiotic polyps in 10^{-6} M DCMU (3,3,4-dichlorophenyl, 1,1-dimethyl urea), for 35–40 days.

Polyps were fed 3× a week to repletion, with 2–4-day-old *Artemia salina* larvae. The cultures were washed and media changed within 24 hr after feeding. Sea water (from the Mediterranean, salinity 37–39‰) was sterilized in an autoclave. Aposymbiosis of the various forms of *Cassiopeia* was verified by fluorescence microscopy. Stock cultures of polyps were maintained at 20° ± 2°C under continuous illumination of 3–5 W/m² from white fluorescent lamps.

Crude homogenates of *Cassiopeia* were prepared from the oral lobes and subumbrellae of adult medusae, using a glass homogenizer. To reinfect aposymbiotic polyps, about 20 μl of fresh homogenate was injected into the polyp's mouth using a drawn-out Pasteur pipette. For "feeding" experiment controls, the same homogenates were heated for 30 min in a water bath at 45°C and stored at −18°C until used. Some homogenates were deep-frozen directly to −18°C without prior heating.

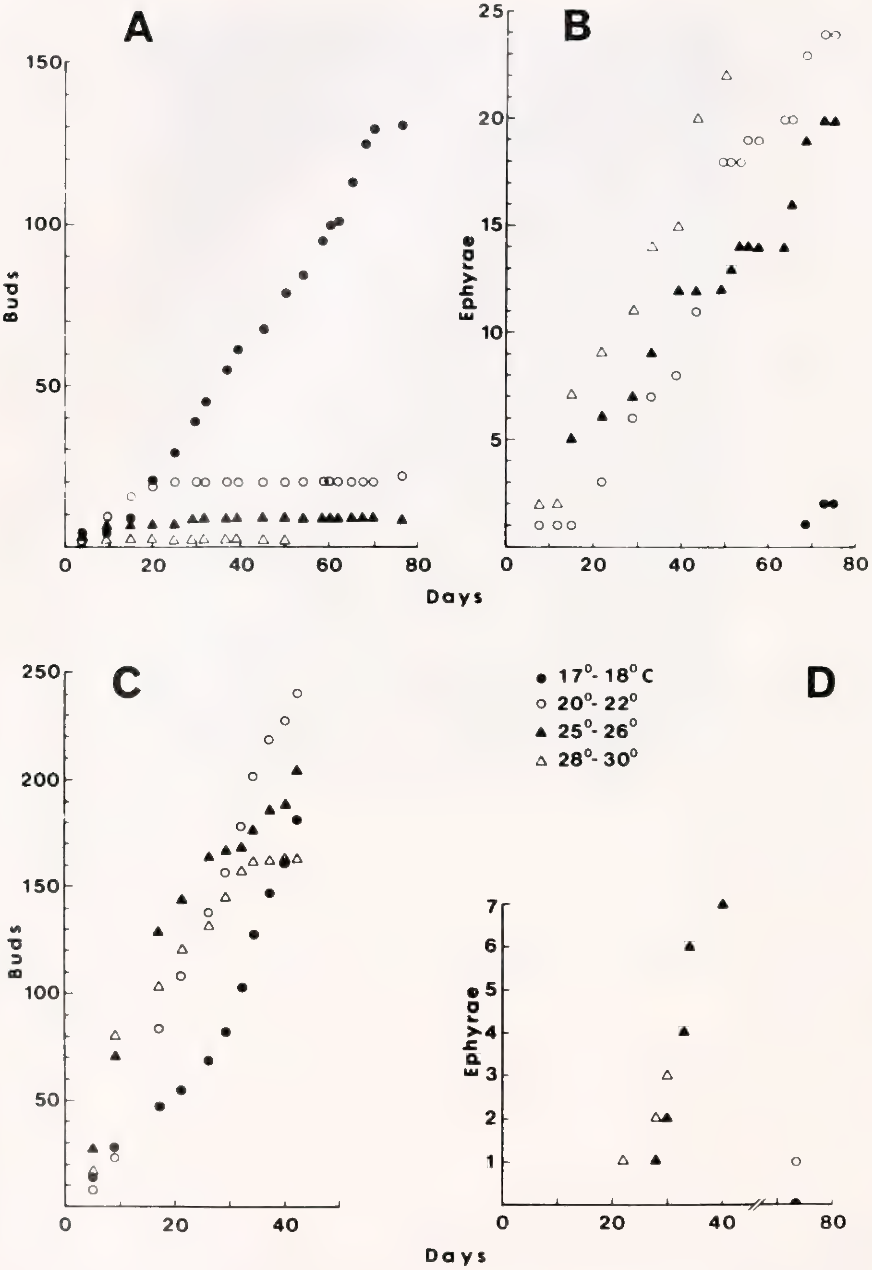


FIGURE 2. Effect of temperature on budding and strobilation of symbiotic and aposymbiotic *C. andromeda*. N = 7. A and B, symbiotic polyps. C and D, aposymbiotic polyps.

RESULTS

Budding and strobilation of symbiotic polyps

Groups of seven symbiotic polyps were placed in crystallizing dishes at four different temperature ranges between 17° and 30°C. The numbers of buds and

TABLE I

Budding and strobilation of C. andromeda *under various conditions.*

Polyps	°C	Buds/polyp	Ephyrae/polyp
Symbiotic* (N = 7)	17°–18°	19.6	0.[illegible]
	20°–22°	3.1	3.4
	25°–26°	1.0	2.8
	28°–30°	0.3	3.1
Aposymbiotic† (N = 7)	17°–18°	25.7	0
	20°–22°	34.3	0.1
	25°–26°	29.1	1.0
	28°–30°	23.1	0.4
Reinfected (N = 10)	20°–22°	2.9	2.0
Injected (N = 10)	20°–22°	62.5	1.0

* Some symbiotic polyps formed up to five ephyrae in succession.

† Aposymbiotic polyps never formed more than one ephyrae. For time-rate of budding and strobilation, see Figures 2 and 4.

ephyrae formed at each temperature were recorded, as shown in Figure 2 (A and B) and Table I. At higher temperatures budding was fully arrested after 20–25 days, and only ephyrae were formed. Strobilation was about equal at all temperatures between 20° and 30°C. Sometimes, budding and strobilation occurred simultaneously, as was noted by Hoffmann *et al.,* (1978).

Budding and strobilation of aposymbiotic polyps

Experimental conditions for aposymbiotic polyps were identical to those described above for symbiotic polyps. Budding was equally frequent at all temperatures tested, and aposymbiotic ephyrae were formed at the higher temperatures (Fig. 2B, C; Table I). In this and similar experiments, a total of 26 aposymbiotic ephyrae (Fig. 3) were obtained from 130 aposymbiotic polyps at temperatures above 25°C. Only two ephyrae were obtained from several hundred aposymbiotic polyps below this temperature.

During strobilation, a yellow-green color appeared in the upper part of the calyx of the aposymbiotic scyphistomae. This color was also observed in the free-swimming young ephyrae, but it disappeared 7–10 days after their release.

Effect of reinfection and homogenate injection

Ten aposymbiotic polyps previously maintained at 18°C were reinfected with zooxanthellae by injection of a fresh homogenate into their coelenterons. The polyps were then maintained at 20°–22°C (Fig. 4A). Within 5 days, endosymbiotic algae could be seen with the naked eye. Strobilation could be seen on about the 10th day, and on the 18th day two ephyrae were released. All buds and ephyrae formed by the reinfected polyps contained zooxanthellae.

Ten similar aposymbiotic polyps maintained at 20°–22°C were injected every 2 days with a homogenate containing heat-killed algae (Fig. 4B). Strobilation was first observed on the 34th day, and the first ephyrae released on the 43rd day. The above experiments are summarized in Table I. In these and in similar "feeding" experiments, a total of 17 ephyrae and 2025 buds were obtained from

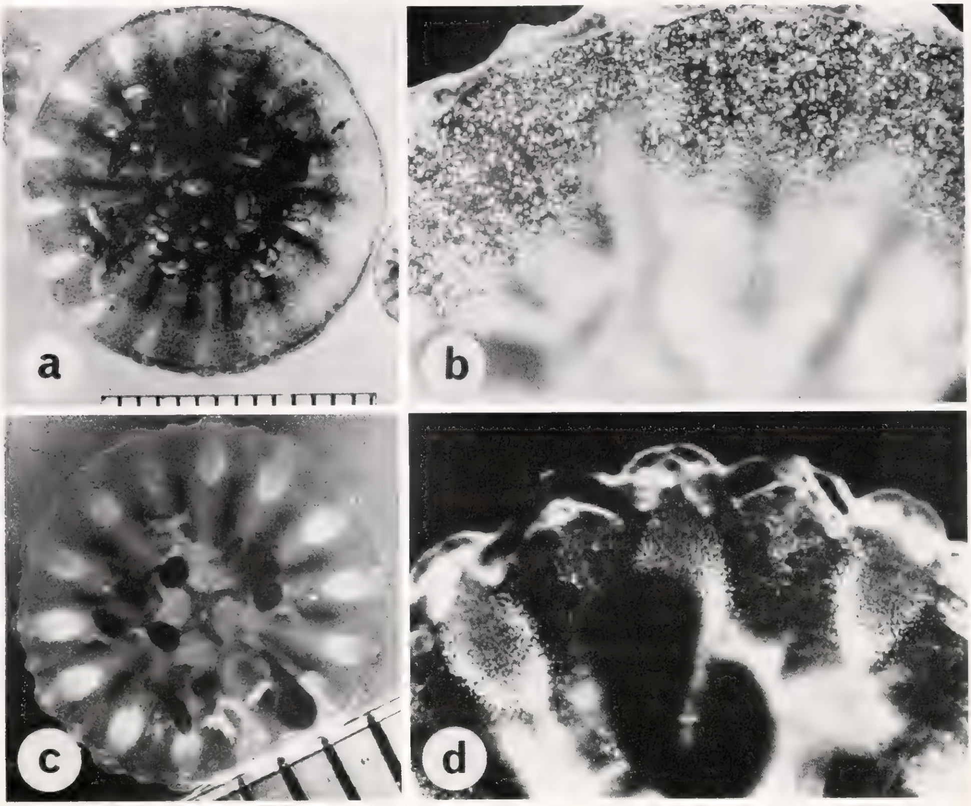


FIGURE 3. Symbiotic and aposymbiotic ephyrae of *C. andromeda.* a. Algae-hosting ephyrae: the dark central area is heavily infected with zooxanthellae. b. Enlarged margin of same: zooxanthellae are seen as bright dots through the transparent umbrella, on dark background. c. Aposymbiotic ephyrae: note transparent central area. The dark oral lobes contain a violet-blue or brown pigment. d. Enlarged margin of same: note transparent umbrella on dark background, and white clusters of nematocytes. In a and c, milimetric scale.

44 injected aposymbiotic polyps. Strobilations were also obtained in experiments in which homogenates with freeze-killed algae were used. There was no reinfection in any of the polyps.

DISCUSSION

This study shows that elevated temperatures and endosymbiotic algae act synergistically to induce strobilation in *C. andromeda.*

The direct effect of temperature on morphogenesis has been observed in both symbiotic and aposymbiotic polyps. In the former, there was a marked preference for bud formation at temperatures of 17°–18°C. At higher temperatures budding ceased completely and only ephyrae were formed. In the aposymbiotic polyps, budding rate was similar at all tested temperatures between 17° and 30°C. Strobilation, however, was definitely temperature dependent, and occurred almost only at above 25°C (Fig. 2, Table I).

Such a requirement for elevated temperatures to induce strobilation has also been reported for the symbiotic scyphozoan medusa *Mastigias papua* (Sugiura, 1965), and for the nonsymbiotic *Chrysaura quinquecirrha* (Loeb, 1972). In both cases, precooling to 20°C for several weeks was required for later strobilation at 22° and 26°C, respectively. The stock cultures of polyps used in our experiments were maintained at 20° ± 2°C, but we did not thoroughly investigate whether such precooling is required also for *C. andromeda.*

The morphogenic effect of endosymbiotic algae was shown by the lower temperatures at which strobilation occurred in the symbiotic polyps, as well as by the relative number of ephyrae and buds formed at each temperature (Table I). The lowest temperature at which aposymbiotic strobilation occurred was 20°–22°C. Symbiotic polyps formed some ephyrae even at 17°–18°C.

We therefore looked for a strobilation-enhancing factor(s) in the endosymbiotic algae.

Reinfection of aposymbiotic polyps enhanced strobilation at 20°–22°C. Twenty ephyrae were formed in 60 days by 10 reinfected polyps (Fig. 4A), *vs.* only one ephyrae formed at the same temperature from seven aposymbiotic polyps after 73 days (Fig. 2D). A similar though less marked effect on strobilation was obtained by injecting preheated or frozen homogenates into aposymbiotic polyps. In one experiment (Fig. 4B), 10 ephyrae were formed by 10 aposymbiotic polyps in 60 days. At the same time, however, 600 buds were formed. This relatively high rate of budding correlates with the high budding rate shown in Figure 2C for aposymbiotic polyps. In all these experiments (Figs. 2, 4), initiation of strobilation was accompanied by a synchronous decrease in budding rate.

The lag time to the release of the first ephyrae was much shorter in the symbiotic polyps than in the aposymbionts (Fig. 2). A similar relation was found between the reinfected polyps and the aposymbionts injected with homogenates (Fig. 4).

From the results obtained in our study, we suggest that morphogenic processes in *C. andromeda* shift from budding to strobilation at relatively higher metabolic rates.

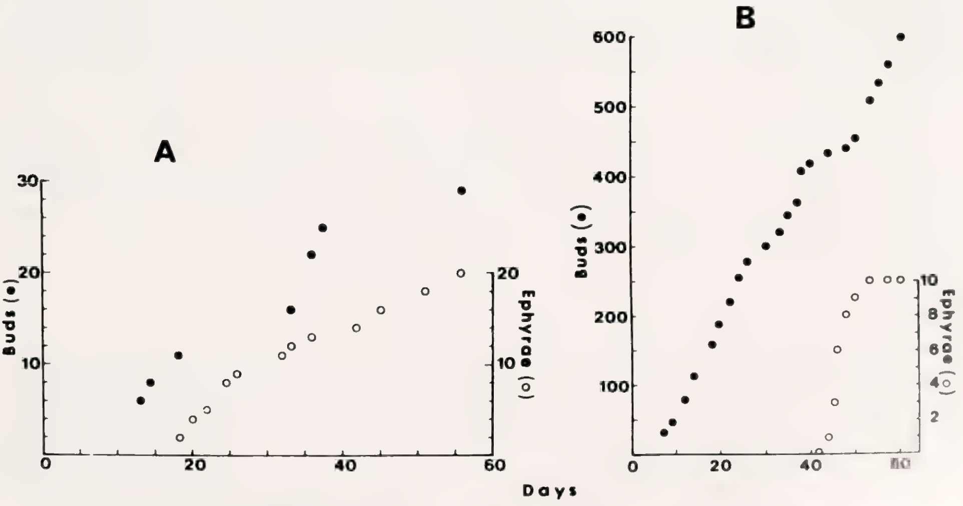


FIGURE 4. Formation of buds and ephyrae by aposymbiotic polyps. N = 10. T = 22°C. A. Polyps reinfected with zooxanthellae on day O. B. Polyps injected with [illegible] homogenate of symbiotic mature medusae.

A higher metabolic rate can be obtained either by a rise in ambient temperature, or by a factor(s) that enhances metabolism. Such a factor is formed by *Cassiopeia* polyps, and it is probable that its accumulation enables strobilation of aposymbionts even at low temperatures. The formation of this factor by *Cassiopeia* is facilitated by a metabolite formed by the zooxanthellae. This algal metabolite is non-specific, as it is present also in non-strobilating symbiotic adult medusae.

Further experiments are required to elucidate quantitatively and qualitatively the characteristics and effects of the proposed factor(s) and its relation to effects of temperature and the metabolites of the algal symbionts.

The ecological implications of the above described combined effects of temperature and endosymbiotic algae can only be speculated upon. In its natural habitat in the Gulf of Eilath, *C. andromeda* can be found along the open coast, as well as in closed lagoons and mangroves. In the open coast the yearly temperature fluctuation is 17°–27°C, but in lagoons and mangroves a range of 13°–33°C has been recorded (Por *et al.*, 1977). Budding would thus be enhanced in winter, while at the higher summer temperatures ephyrae would be formed. In the sea, no aposymbiotic *C. andromeda* have yet been reported. By the ease at which algal infections occur in these polyps, it is doubtful whether aposymbiotic specimens ever occur in their natural habitat.

We do not know yet if aposymbiotic ephyrae can grow into sexually mature medusae, and thus complete an aposymbiotic life cycle in this species. In preliminary experiments we obtained a twofold increase in diameter of aposymbiotic ephyrae (from 5.1 to 11.5 mm), accompanied by normal morphological development.

Our observations on aposymbiotic strobilation in *C. andromeda* raise some questions with regard to the evolution of obligatory symbioses. Loss of capacity in a host to complete its life cycle in absence of its symbionts would indicate an evolutionary genetic integration in the sense described by Margulis (1976). It would thus be possible to claim that we have at our hands a new species, made up respectively of animal and algal tissues. Such an integration, however, should express itself also in the capability of the host to directly inherit its endosymbionts through the germ cells. In *C. andomeda,* as well as in *Mastigias papua, Amphiscolops langerhansi*, and *Convoluta roscoffensis*, this is not the case. Aposymbiotic larvae are formed by these species, and the symbiotic algae are acquired anew at each generation.

On the basis of our results that strobilation in *C. andromeda* is not dependent upon a symbiont, *vs.* the hypothesized obligatory dependence of the above species on their respective endosymbiotic algae, it would be of interest to reexamine the algae for the extent of host-symbiont integration. Such a study would add to our understanding of the effect of symbiosis on evolution.

SUMMARY

The role of the zooxanthellae in the life cycle of *Cassiopeia andromeda* was reexamined. Symbiotic and aposymbiotic polyps were maintained at various temperatures between 17° and 30°C, and numbers of buds and ephyrae formed at each temperature were recorded.

The number of buds formed by the symbiotic polyps was inversely related to temperature. After 20 days at above 20°C, only ephyrae were formed.

In aposymbiotic polyps, buds were formed at all temperatures tested. Strobilation occurred above 25°C, and aposymbiotic ephyrae were obtained. Aposymbiotic

polyps reinfected with zooxanthellae formed ephyrae at 22° C. A similar effect was obtained by repeatedly injecting aposymbiotic polyps with heat- or cold-inactivated homogenate derived from adult medusae. No reinfection occurred in the latter.

It is concluded: 1. At higher metabolic rates, strobilation supersedes budding in *C. andromeda.* 2. A higher metabolic rate is obtained at elevated temperatures, and by a biotrophic effect of the symbiotic zooxanthellae. 3. Symbiotic zooxanthellae are not essential for strobilation in *C. andromeda.*

LITERATURE CITED

COOK, C. B., 1972. Benefit to symbiotic zoochlorellae from feeding by green hydra. *Biol. Bull.,* **142**: 236–242.

HOFFMANN, D. K., R. NEUMANN, AND K. HENNE, 1978. Strobilation, budding and initiation of scyphistoma morphogenesis in the rhizostome *Cassiopeia andromeda (Cnidaria: Scyphozoa). Mar. Biol.* **47**: 161–176.

LOEB, M., 1972. Strobilation in the Chesapeake Bay sea nettle *Chrysaura quinquecirrha.* I. The effects of environmental temperature changes on strobilation and growth. *J. Exp. Zool.* **180**: 279–292.

LUDWIG, F. D., 1969. Die zooxanthellen bei *Cassiopeia andromeda* Eschscholz 1829 (polypstadium) und ihre bedeutung für die strobilation. *Zool. Jb. Abt. Anat. Ontog. Tiere* **86**: 238–277.

MARGULIS, L., 1976. Genetic and evolutionary consequences of symbiosis. *Exp. Parasitol.* **39**: 277–349.

POR, F. D., I. DOR, AND A. AMIR, 1977. The mangal of Sinai: Limits of an ecosystem. *Helgol. Wiss. Meeresunters.,* **30**: 295–314.

PROVASOLI, L., T. YAMASU, AND I. MANTON, 1968. Experiments on the resynthesis of symbiosis in *Convoluta roscoffensis* with different flagellate cultures. *J. Mar. Biol. Assoc. U. K.* **48**: 465–479.

RICHMOND, M. H., AND D. C. SMITH, Eds., 1979. *The cell as a habitat.* The Royal Society, London.

SMITH, D. C., L. MUSCATINE, AND D. H. LEWIS, 1969. Carbohydrate movement from autotrophs to heterotrophs in parasitic and mutulistic symbiosis. *Biol. Rev.* **44**: 17–90.

SUGIURA, Y., 1964. On the life history of rhizostome medusae. II. Indispensability of zooxanthellae for strobilation in *Mastigias papua. Embryologia,* **8**: 223–233.

SUGIURA, Y., 1965. On the life history of rhizostome medusae. III. On the effects of temperature on the strobilation of *Mastigias papua. Biol. Bull.,* **128**: 493–496.

TAYLOR, D. L., 1971. On the symbiosis between *Amphidinium klebsii (Dinophyceae)* and *Amphiscolops langerhansi (Turbellaria: Acoela). J. Mar. Biol. Assoc. U. K.* **51**: 301–313.

Reference: *Biol. Bull.*, **159**: 402–417. (October, 1980)

THE BEHAVIORAL BASIS OF LARVAL RECRUITMENT IN THE CRAB *CALLINECTES SAPIDUS* RATHBUN: A LABORATORY INVESTIGATION OF ONTOGENETIC CHANGES IN GEOTAXIS AND BAROKINESIS[1]

S. D. SULKIN, W. VAN HEUKELEM, P. KELLY, AND L. VAN HEUKELEM

Horn Point Environmental Laboratories, Center for Environmental and Estuarine Studies, University of Maryland, Cambridge, Maryland 21613

Estuarine invertebrates that have planktonic larvae must maintain their populations in the face of net seaward flow of water. One mechanism for this is active retention within the estuary of sufficient offspring to at least replace the population (Sandifer, 1975; Scheltema, 1975).

Retention mechanisms that combine behavioral adaptations with characteristic estuarine circulation have been described for larvae of several estuarine invertebrates, including barnacles and mud crabs (Bousfield, 1955), oysters (Carriker, 1951; Wood and Hargis, 1971) and decapods (Sandifer, 1975). However, it is unlikely that all planktonic larvae will be retained in the face of net downstream flow of water. Indeed, larvae of many estuarine crabs are present in the waters of the continental shelf off the east coast of North America (Nichols and Keney, 1963; Sandifer, 1973; Dudley and Judy, 1971). Among the most common are species of the genus *Callinectes,* including the blue crab *Callinectes sapidus* Rathbun (Sandifer, 1975; Goy, 1976).

C. sapidus inhabits entire estuaries as an adult, but spawns only in high salinity regions near estuary mouths (Hopkins, 1943; Van Engel, 1958). These circumstances increase the probability of loss of larvae from the estuary. It is critical to an understanding of the recruitment process, and hence population dynamics, to determine whether larvae exported from estuarine spawning grounds to ocean waters represent a significant loss from the adult habitat and, if so, whether they may be recruited back to it in significant numbers.

Mechanisms for larval retention within an estuary or exchange between an estuary and its adjacent coastal region depend upon characteristic circulation (Pritchard, 1952; Bousfield, 1955; Scheltema, 1975). Because currents vary in direction and rate with depth, vertical distribution of larvae is significant in determining their ultimate destinations. For some crab species, there is evidence that vertical distribution of larvae varies predictably with age (Bousfield, 1955; Sandifer, 1975; Goy, 1976). There is further evidence that these vertical distribution patterns result from oriented movement controlled by behavioral responses to exogenous stimuli (Sulkin, 1973, 1975; Forward and Costlow, 1974; Ott and Forward, 1976; Latz and Forward, 1977).

Any *C. sapidus* adaptation for retention or exchange would be likely to be manifest in behavioral patterns that regulate vertical distribution through ontogeny. We have examined the behavioral responses of *C. sapidus* at various larval stages to stimuli likely to influence vertical movement. We report here the results of experiments on orientation and swimming rate in response to gravity, hydrostatic pressure, temperature, and salinity. The results are evaluated in terms of their effects on vertical distribution of *C. sapidus* larvae throughout their ontogeny and consequent patterns of dispersal of this species in the estuarine and coastal marine environments.

---

[1] Contribution No. 1094 from the Center for Environmental and Estuarine Studies, University of Maryland.

MATERIALS AND METHODS

Larval culture

Ovigerous blue crabs were obtained from lower Delaware Bay and were taken to the laboratory with egg masses intact. Eggs were stripped from the females when they reached the black eyespot stage, or immediately if they were collected at that stage. Approximately 500 eggs from a single female were then placed in each of several 125 ml Erlenmeyer flasks containing 50 ml of filtered seawater at 30‰ salinity (S) and 20°–30°C. Culture water contained either penicillin (60 mg/l) and streptomycin (50 mg/l) or chloramphenicol at 5 mg/l. Unpublished data show that use of antibiotics improves egg and larval survival without altering larval behavior. Eggs were transferred to fresh medium every second day.

Upon hatching, larvae from a given female were placed in several glass culture dishes containing 100 ml of 30‰ S seawater. The standard culture temperature was 25°C, although experiments occasionally dictated acclimation of larvae to 15°C. Each dish initially contained approximately 200 zoeae.

Zoeae were transferred to fresh medium daily and fed either the rotifer *Brachionus plicatilis* Muller (during the first 14 days of development) or freshly-hatched nauplii of the brine shrimp *Artemia salina* L. (after day 15; Sulkin, 1978). Records of mortality and molting were kept.

Samples of larvae were removed for testing as experiments dictated. In order to avoid complicating effects of rhythmicity in locomotory activity (Sulkin *et al.*, 1979), all behavior experiments were run during afternoon hours. Experiments were conducted on zoeal stages I, IV, and VII.

Sinking rates

Larvae of various stages were narcotized using 3% ethyl carbamate. Individuals were timed as they sank vertically through a 10-cm-long space marked off midway along a vertical cylinder filled with seawater (30‰ S; 25°C). At least 50 individuals from three different broods were tested. Data are presented as mean sinking rates (cm/sec.). Differences among stages were tested by analysis of variance.

Geotaxis

Typically the presence and sign of geotaxis in crab larvae has been determined by noting changes over time in net distribution of organisms placed in a vertically oriented test chamber in total darkness (Sulkin, 1973; Ott and Forward, 1976; Latz and Forward, 1977). Oriented movement upward has been termed negative geotaxis; downward, positive geotaxis.

To account more effectively for non-oriented or random movement that occurs simultaneously with oriented movement or geotaxis, the following experimental design was used. Thirty to fifty larvae were drawn at random from the culture and divided between two test chambers, one positioned vertically; the other horizontally. Each chamber was constructed of 3-mm-thick transparent lucite, measured 25 × 5 × 5 cm, and was marked off into four sections of equal length. Larvae were placed at the bottom of the vertical experimental chamber and at one end of the horizontal control chamber. At 10-min intervals, the larvae in each quadrant were counted. A dim deep-red light was used to silhouette the larvae when counts were made. This procedure did not appear to disrupt the positions of larvae. Change in distribution over time in both horizontal and vertical chambers was analyzed by construction of a "kite" diagram, which shows the proportion of total sample in each quadrant.

Movement of larvae away from the end of the horizontal chamber was considered random or non-oriented and thus served as a control against which to compare the distributional change which occurred in the vertical chamber. Admittedly, the control was imperfect in that it did not account for sinking, which occurred intermittently in the vertical chamber. Nevertheless, movement along the axis from the initial point source in the vertical chamber exceeding that for the horizontal chamber can be attributed to oriented response—in this case, negative geotaxis. On the other hand, movement in the horizontal control exceeding that in the vertical tank can be attributed to positive geotaxis. If there is no difference between the two, the presumption is that no oriented response can be attributed to geotaxis.

Distributions after 30 min were analyzed. This period proved long enough to separate oriented from random movement while providing for stable distribution in the vertical chamber and random dispersal in the horizontal chamber.

Larvae used in these experiments were acclimated to darkness for at least 2 hr prior to testing and not fed during the experiment. For each larval stage, experiments were conducted at 25, 30, and 35‰ S in the following fashion. A group of larvae was first tested at 30‰ S. Then they were placed in test chambers which contained either 25‰ S seawater or 35‰ S seawater, and distribution shift experiment was repeated. A minimum of three replicates with offspring from three different crabs was conducted for each salinity.

The experimental design permitted statistical analysis as well as the qualitative analysis typically used in behavioral experiments. For each experimental and control replicate, an average distribution at 30 min ("mean position value") was calculated by assigning weights from 0–3 to the quadrants (the end to which the larvae were initially added = 0), multiplying the weights by the number of larvae in the quadrants, and dividing the product by the total number of larvae. The mean position value is a quantitative descriptor of the distribution and provides a commonly derived set of data for all experimental and control replicates. For each salinity, the mean position values for all experimental replicates were compared against those for all control replicates using the non-parametric Mann-Whitney U test.

Swimming rate

Figure 1 shows the apparatus used to measure larval swimming rates. A sample of larvae from a particular brood at a specified stage of development was placed in the behavior chamber. Larvae were attracted to one end of the chamber by means of a broad-spectrum light (75 W/m^2). The larvae were then induced to swim along the axis of the tank by reversing the direction of the light. Approximately 20 individuals were timed during each experiment as they swam through a 5-cm-long section of the chamber.

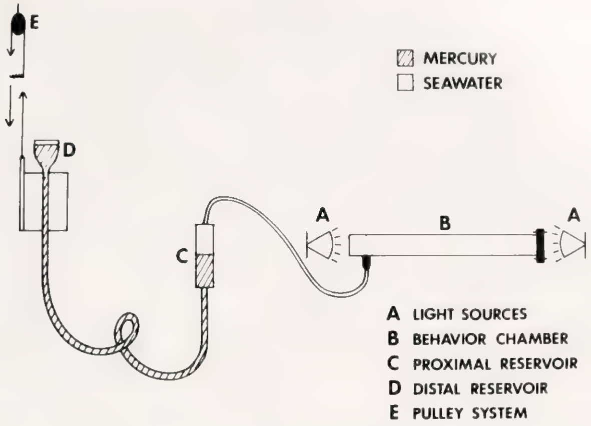


FIGURE 1. Diagrammatic representation of apparatus used to measure swimming rate as a function of hydrostatic pressure.

The effect of pressure on swimming rate was measured by repeating the procedure described above at various pressure increments. Pressure was increased by raising the distal end of the mercury manometer shown in Figure 1 (Sulkin, 1973). In nature, pressure increases with depth at a rate of 1 atm per 10 m of water. In one set of experiments the pressure increments used were 0, 20, 40, and 60 cm Hg (0, 0.26, 0.52, 0.78 atm, respectively). In a second set of experiments, the increments were 0, 1, 2, and 3 atm. The pressures were tested sequentially. Experiments were repeated with larvae from several broods, with total sample sizes ranging from 70 to 140 individuals.

The effects of temperature and salinity on swimming rate were measured by repeating the procedure described above. Larvae were acclimated to the specified temperature or salinity for 24 hr before testing.

RESULTS

Sinking rates

Mean passive sinking rates are shown for zoeal stages I, IV, and VII in Figure 2. Analysis of variance confirms a significant increase in sinking rate from the hatching stage to zoeal stage VII ($P < 0.001$).

Geotaxis

Figure 3 shows the distributions at 30 min for all replicates conducted on stage I larvae at the three salinities. The mean distribution for each salinity is shown at the bottom of Figure 3. It is apparent that negative geotaxis is strong and pervasive in stage I larvae. It also appears that a 5‰ salinity change between 25 and 35‰ S has little, if any, effect upon the presence or sign of geotaxis in zoeal stage I.

Statistical analysis supports these conclusions. The results in Table I indicate that the experimental mean position values were higher than the control in every case and that the differences were significant in all three salinities.

Figure 4 shows the distributions at 30 min for all replicates conducted on stage IV larvae at three salinities. The mean distribution for each salinity is

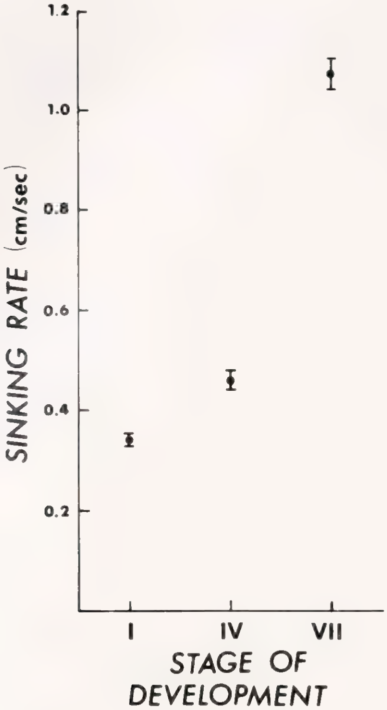


FIGURE 2. Mean passive sinking rates for zoeal stages I, IV, and VII of *Callinectes sapidus.* Vertical bars represent ± 1 standard error.

TABLE I

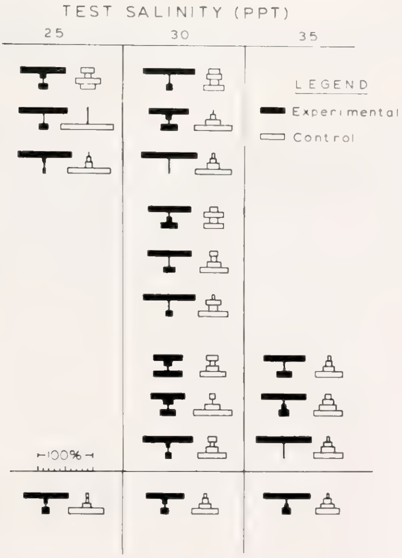
Analysis of relative distributions between vertical experimental and horizontal control tests for stage I larvae at three salinities. Calculation of mean position value described in text.

Salinity (‰)	Mean position value		Mann-Whitney *U* test probability
	Experimental	Control	
25	2.50	1.17	
	2.50	0	<0.05
	2.95	0.33	
30	2.85	1.31	
	2.11	0.45	
	3.00	0.45	
	2.32	1.28	
	2.40	0.76	<0.001
	2.76	0.76	
	1.61	1.05	
	2.10	0.55	
	2.80	0.80	
35	2.35	0.85	
	2.45	0.85	<0.05
	3.00	0.68	

shown at the bottom of Figure 4. In the tests at 30‰ S, there was considerable variability in responses shown in experimental replicates. Although it appears that negative geotaxis is exhibited by a portion of the sample in each replicate, there is also an indication in all replicates that a portion of the sample may be responding with a positive geotaxis. A reduction in salinity from 30 to 25‰ S decreases the proportion of the sample responding with a negative geotaxis while an increase from 30 to 35‰ S increases the proportion exhibiting negative geotaxis. The fourth stage may be in a transition period during which the sign of geotaxis is variable and is sensitive to salinity change.

The data in Table II show that at 25‰ S, mean position values of controls exceeded experimental values in every case, although a Mann-Whitney *U* test indicates no significant difference. At 30‰ S, however, experimental mean position values exceeded controls in 9 of 10 replicates, although again the differences were not statistically significant. At 35‰ S, experimental values significantly exceeded controls for all replicates.

Figure 5 shows the distribution at 30 min for all replicates conducted on stage VII larvae at the three salinities. The mean distribution at each salinity is shown at the bottom of Figure 5. Dispersal from a point source (bottom quadrant) in the vertical chamber is clearly lower than that attributable to random movement alone. We interpret these results as indicating positive geotaxis in the vast majority of individuals. A 5‰ S increase or decrease from 30‰ S appears to have little effect.



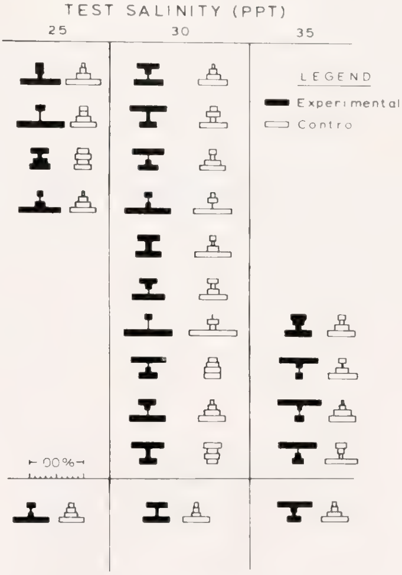


FIGURE 3. (Left.) Geotaxis in *Callinectes sapidus* zoeal stage I. Proportional distributions of larvae among the four sections of experimental and control tanks 30 min after initiation of experiments for all replicates tested at each salinity. The diagrams below the bottom horizontal line represent the mean distributions of all replicates at each salinity. Experimental design described in text.

FIGURE 4. (Right.) Geotaxis in *Callinectes sapidus* zoeal stage IV. Proportional distribution of larvae among the four sections of experimental and control tanks 30 min after initiation of experiments for all replicates tested at each salinity. The diagrams below the bottom horizontal line represent the mean distributions of all replicates at each salinity. Experimental design described in text.

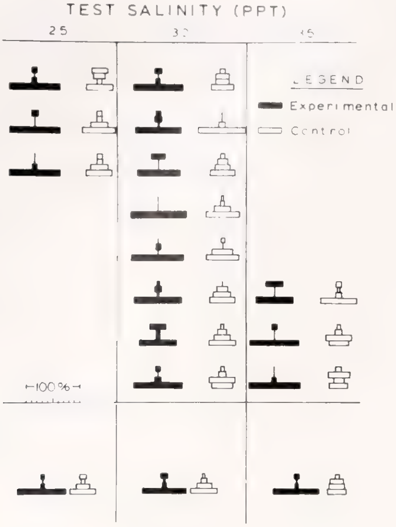


FIGURE 5. Geotaxis in *Callinectes sapidus* zoeal stage VII. Proportional distribution of larvae among the four sections of experimental and control tanks 30 min after initiation of experiments for all replicates tested at each salinity. The diagrams below the bottom horizontal line represent the mean distributions of all replicates at each salinity. Experimental design described in text.

The data in Table III show that experimental mean position values are lower than control values for all replicates at 25‰ S, for six of eight replicates at 30‰ S, and for two of three replicates at 35‰ S. The Mann-Whitney *U* test

TABLE II

Analysis of relative distributions between vertical experimental and horizontal control tests for stage IV larvae at three salinities. Calculation of mean position value described in text.

Salinity (‰)	Mean position value		Mann-Whitney *U* test probability
	Experimental	Control	
25	0.55	0.61	=0.10
	0.48	1.04	
	1.33	1.36	
	0.32	0.91	
30	1.30	0.72	>0.10
	2.00	1.03	
	1.82	0.84	
	0.37	1.64	
	1.44	0.64	
	0.92	0.90	
	0.40	0.25	
	1.87	1.26	
	1.04	0.84	
	1.95	1.70	
35	1.26	0.90	=0.05
	2.65	0.78	
	2.48	0.72	
	2.20	0.95	

Table III

Analysis of relative distributions between vertical experimental and horizontal [illegible] tests for stage VII larvae at three salinities. Calculation of mean position value described in text.

Salinity (‰)	Mean position value		Mann-Whitney U-test probability
	Experimental	Control	
25	0.19	1.25	<0.05
	0.25	0.55	
	0.06	2.38	
30	0.33	1.05	<0.05
	0.42	0.10	
	0.67	0.94	
	0	0.56	
	0.08	0.48	
	0.39	0.75	
	0.94	0.80	
	0.25	0.86	
35	1.00	0.72	>0.05
	0.30	0.86	
	0.05	1.24	

indicates significant differences between distributions in vertical and horizontal tanks at 25 and 30‰ S, but not at 35‰ S.

Barokinesis

Because early larval stages of estuarine crabs are sensitive to small increases in hydrostatic pressure (Sulkin, 1973; Bentley and Sulkin, 1977; Wheeler and Epifanio, 1978), initial experiments were designed to determine response of blue crab larvae to small pressure increments. The data in Figure 6 show mean

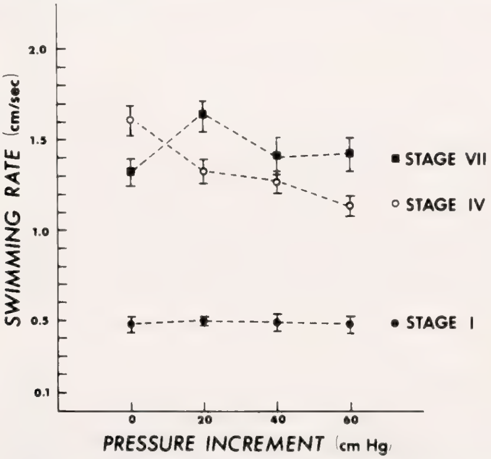
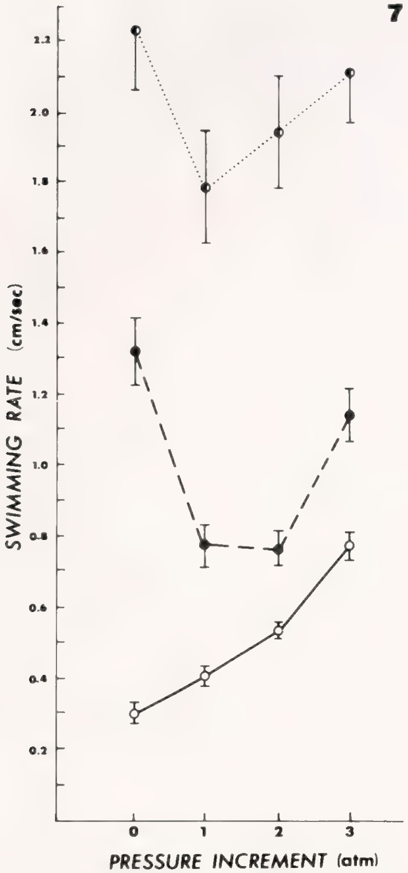


Figure 6. Mean swimming rates (± 1 standard error) of *Callinectes sapidus* zoeal stages I, IV, and VII in pressure increments up to 60 cm Hg (0.78 atm), in seawater of [illegible] C and 30‰ S.



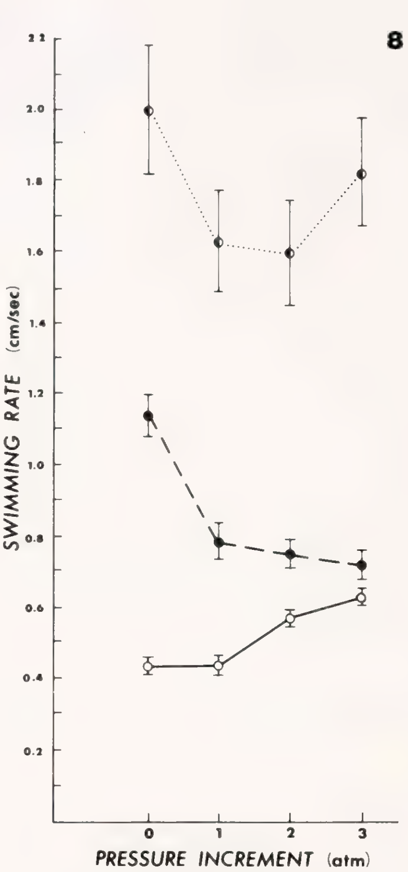


FIGURE 7. (Left.) Mean swimming rates (± 1 standard error) of *Callinectes sapidus* zoeal stage I (open circle), stage IV (closed circle), and stage VII (half-closed circle) as a function of hydrostatic pressure in filtered seawater at 25°C and 25‰ S.

FIGURE 8. (Right.) Mean swimming rates (± 1 standard error) of *Callinectes sapidus* zoeal stage I (open circle), stage IV (closed circle), and stage VII (half-closed circle) as a function of hydrostatic pressure in filtered seawater at 25°C and 30‰ S.

swimming rates of zoeal stages I, IV, and VII in small pressure increments up to 60 cm Hg (0.78 atm). The experiments were conducted at 25°C, 30‰ S. In contrast to results for other estuarine species, stage I blue crab larvae do not respond to small increases in hydrostatic pressure. Two-way analysis of variance (development stage × pressure) indicates significant difference in swimming rate due to development stage ($P < 0.005$), but no differences due to pressure ($P > 0.05$). The interaction term, however, was significant ($P < 0.005$), probably due to the trend in zoeal stage IV for swimming rate to decrease with increased pressure.

Mean swimming rates for the same three stages at pressure increments up to 3 atm are shown in Figures 7, 8, and 9 at salinities of 25, 30, and 35‰ S, respectively. Larvae were acclimated to the test salinity. Temperature was kept at 25°C. Three-way analysis of variance showed significant differences due to development stage ($P < 0.001$), pressure increment ($P < 0.005$), and salinity ($P <$

0.001). The only significant two-way interaction was stage × pressure ($P < 0.025$). Higher salinities have little effect on stage I larvae, but depress swimming rate in zoeal stages IV and VII. As the significant interaction term suggests, the effect of increased pressure is stage dependent. There is an increase in swimming rate as pressure increment exceeds one atm in zoeal stage I (high barokinesis) and a reduction in swimming rate with pressure increase in zoeal stages IV and VII (low barokinesis).

To determine the effect of reduced temperature on barokinesis, swimming rates were measured at 15°C at ambient pressure and at 3 atm pressure increment (Fig. 10). A two-way analysis of variance was conducted for each stage, testing temperature against pressure. Appropriate data collected at 0 and 3 atm increments at 25°C, 30‰ S, were used in the analyses. For zoeal stage I, no significant dif-

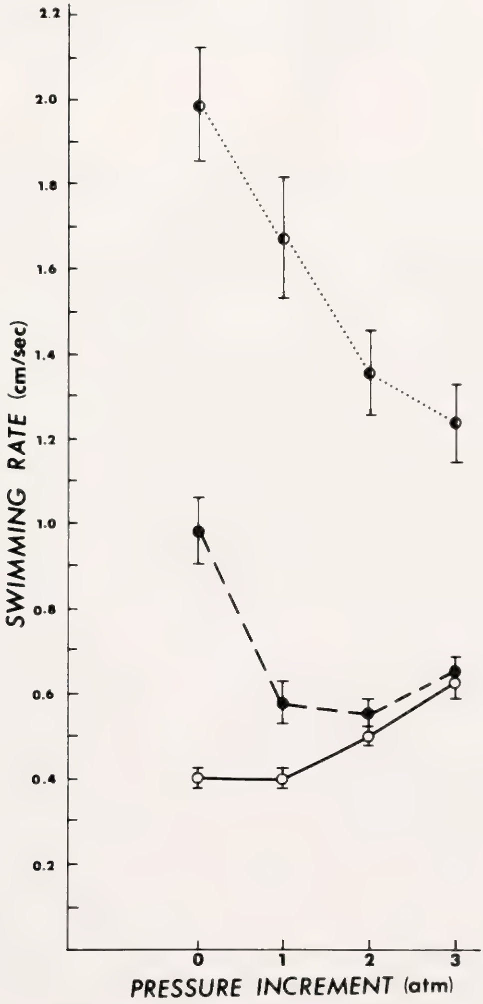


FIGURE 9. Mean swimming rates (± 1 standard error) of *Callinectes sapidus* zoeal stage I (open circle), stage IV (closed circle), and stage VII (half-closed circle) as a function of hydrostatic pressure in filtered seawater at 25°C and 35‰ S.

ferences were attributable to temperature ($P > 0.05$). Reduced temperature significantly reduces swimming rate only in zoeal stages IV ($P < 0.001$) and VII ($P < 0.001$), with significant interaction with pressure only in zoeal stage IV ($P < 0.01$). However, the reduction in temperature appears to moderate both high and low barokinesis to some degree in all developmental stages.

Data reported in Figures 8 and 9 indicated that acclimation to higher salinity reduces swimming rate in zoeal stages IV and VII. Shown in Figure 11 are the results of an experiment designed to determine the effect of a 10‰ S increase on larvae acclimated to the lower salinity. A two-way analysis of variance indicated significant interaction between development stage and salinity ($P < 0.005$) with stages significant ($P < 0.001$) and salinity not significant ($P > 0.05$). Because of the significant interaction, a series of one-way analysis of variance tests were conducted for each stage. The results indicated a significant difference in zoeal stage I ($P < 0.05$), but no significant differences in either zoeal stage IV or VII. Thus a 10‰ S increase from that of acclimation increases swimming rate in zoeal stage I, but has little effect on zoeal stages IV and VII.

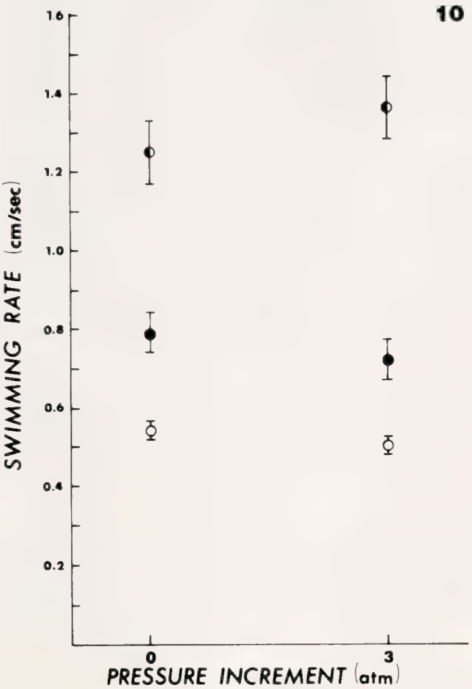
DISCUSSION

Thorson (1950) and others have suggested there is adaptive value in a changing pattern of vertical distribution in developing planktonic larvae of benthic species. For example, migration towards the surface in early larval stages may enhance dispersal, provide for a reliable source of food and an optimum of temperature and light, and reduce immediate competition with adult members of the population (Thorson, 1950; Mileikovsky, 1972). However, the late stages of benthic species must move to the bottom for metamorphosis or post-metamorphic development.

In order to understand the regulation of vertical distribution through ontogeny, it is necessary to investigate the component factors which control vertical movement and how these factors change as development proceeds. The vertical position of a negatively buoyant planktonic animal is the net result of a complex combination of factors, including its sinking rate, the direction of active orientation, swimming rate, and the proportion of time spent sinking and swimming. Changes in vertical position among various planktonic larval stages can be due to changes in one or more of these factors as development proceeds.

Orientation and swimming are highly responsive to external stimuli. Because in nature many of these stimuli are variable and unpredictable, it is difficult to generalize about the presence of adaptive behavioral patterns. However, behavioral adaptations which produce a characteristic pattern of vertical distribution through ontogeny probably would evolve in response to ubiquitous and predictable selective pressures. If this is true, it may be necessary to look only at behavioral responses to such conservative stimuli to detect the presence of a characteristic adaptive pattern of vertical distribution. The results reported here for response to gravity and hydrostatic pressure suggest that this is the case for *C. sapidus*.

The finding of negative geotaxis in the first larval stage is consistent with earlier reports for crab larvae (Sulkin, 1973; Latz and Forward, 1977). Negative geotaxis tends to orient the larvae so that active swimming will result in upward movement. Moreover, as larvae move deeper, increased pressure will result in increased swimming rate. The combination of negative geotaxis and high barokinesis thus produces a depth regulatory mechanism (Sulkin, 1973). Sensitivity to pressure change is common in early larval instars of crabs (Hardy and Bain-



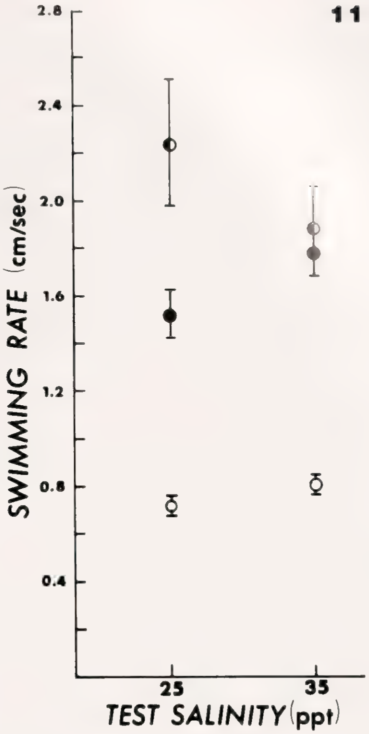


FIGURE 10. (Left) Mean swimming rate (± 1 SE) of *Callinectes sapidus* zoeal stage I (open circle), stage IV (closed circle), and stage VII (half-closed circle) at ambient pressure and at a pressure increment of 3 atm in filtered seawater at 30‰ S and 15°C.

FIGURE 11. (Right) Mean swimming rate (± 1 SE) of *Callinectes sapidus* zoeal stage I (open circle), stage IV (closed circle), and stage VII (half-closed circle) in test salinities of 25‰ and 35‰ (25°C). All larvae acclimated to 25‰ S and 25°C.

bridge, 1951; Rice, 1964, 1966; Sulkin, 1973; Bentley and Sulkin, 1977; Wheeler and Epifanio, 1978). However, the high threshold for pressure sensitivity in *C. sapidus* zoeae suggests that the pressure mechanism is not activated in shallow systems. On the other hand, the increase in swimming rate which occurs with a salinity increase (Fig. 11) would complement the pressure mechanism in a stratified, deep system or substitute for it in a more shallow one.

If stage I larvae descend from surface waters, they will encounter increased pressure, increased salinity, and decreased temperature. Zoeal stage I responds to gravity, pressure, salinity, and temperature in ways which will produce upward movement. Stage I larvae thus exhibit behavioral adaptations likely to promote maintenance of vertical position high in the water column.

By the fourth larval instar, changes in behavior have occurred which are likely to produce deeper vertical position. Fourth stage zoeae have a sinking rate greater than that of zoeal stage I and are in a period of transition between negative and positive geotaxis. In any sample of stage IV larvae, some individuals will exhibit negative geotaxis and others positive geotaxis. Furthermore, the sign of geotaxis in individual larvae is sensitive to salinity change. A 5‰ reduction in salinity induces increased incidence of positive geotaxis, whereas a 5‰ S increase induces increased incidence of negative response. This is similar to responses reported by Latz and Forward (1977) for *Rhithropanopeus harrisii* larvae. In a stratified estuary or the near-shore marine environment, these complex responses help regulate depth. More larvae become positively geotactic as their development proceeds. As these larvae swim downward, however, they will encounter increased salinity, which tends to reverse the sign of geotaxis. In this way, intermediate stages will tend to migrate down from surface waters, but maintain their position up in the water column.

Unlike stage I larvae, the fourth stage does not respond to a salinity increase by increasing swimming rate, a response which, in combination with reversal of geotaxis, would carry them upward. Indeed, as fourth stage larvae become acclimated to higher salinities, their swimming rate drops. It also drops with reduction in temperature or with increase in pressure. These adaptations help insure that once fourth stage larvae move downward from surface waters, they will be retained at depth.

By the seventh, or final, instar the transition to positive geotaxis is complete. Positive geotaxis is pervasive and insensitive to salinity change. Our results cannot be attributed either to inactivity or to increased sinking rate in zoeal stage VII. Random movement of stage VII larvae, as measured in the horizontal controls, exceeds that for zoeal stage I and is equal to that for zoeal stage IV. Although sinking rate increases 3.2-fold between zoeal stages I and VII, swimming rate increases 4.4-fold (measured at 30‰ S, ambient pressure). The positive geotaxis reported here is in contrast to the persistent negative geotaxis reported for zoeae of other estuarine species (Sulkin, 1973). However, the last zoeal stage of the mud crab *Rhithropanopeus harrisii* shows positive geotaxis (Latz and Forward, 1977) as does the megalopa of the crab *Leptodius floridanus* (Sulkin, 1973).

The same factors which reduce swimming rate in stage IV larvae as they move deeper are operative in zoeal stage VII. Swimming rate does not change with an increase in salinity, but does decrease as larvae become acclimated to higher salinity, or are subjected to increased pressure or reduced temperature. These responses are likely to produce a deep vertical position in zoeal stage VII.

Thus behavioral response to pressure and gravity, as modified by temperature and salinity, provide the basis for differential vertical distribution during larval development of *C. sapidus*. Early stage larvae that enter surface waters, either by hatching in shallow areas or by migrating upward, are likely to stay near the surface. Changes in behavioral response will stimulate larvae in later zoeal stages to move deeper. Behavioral evidence that suggests a prevalence of early stages in surface waters and late stages in deep water is consistent with field evidence (Sandifer, 1975; Goy, 1976).

This adaptive pattern of vertical distribution has predictable conseqences to larval dispersal in an estuarine and near-shore marine environment. The proximity of spawning grounds to the estuary mouth and the complementary behavioral adaptations which insure the presence of early stages in seaward-flowing surface waters will result in tremendous losses of larvae from the estuarine habitat. However, a mechanism for recruitment back to the estuary exists. Deep waters of the continental shelf along the Atlantic coast of North America drift landward (Bumpus, 1965; Harrison *et al.*, 1967; Beardsley *et al.*, 1976), with particularly noticeable landward components at the mouths of major estuaries (Harrison *et al.*, 1967). Scheltema (1975) suggests that larvae positioned in this deep, landward-flowing drift would be carried shoreward and perhaps into an estuary. Offshore recruitment thus may be particularly significant in highly stratified estuaries with significant upstream non-tidal drift along the bottom.

The adaptive vertical distribution pattern reported here will, of course, be modified by the vagaries of the environment. Furthermore, the megalopa stage undoubtedly is also important in recruitment and may exhibit different behavioral responses from those reported here for late zoeal stages (Naylor and Isaac, 1973). Nevertheless, a mechanism for larval exchange between the estuary and the open sea exists. This mechanism is fundamentally the same as that for retention, described for other estuarine species (Bousfield, 1955; Wood and Hargis, 1971), and results from a combination of hydrographic features common in estuarine and coastal marine environments and behavioral responses that produce an adaptive pattern of differential vertical distribution during the period of larval development.

C. sapidus differs from other estuarine species in that its offspring originate close to the estuary mouth. As a result, the probability of retention is reduced and the potential significance of offshore recruitment is increased. In stratified estuaries, population dynamics of *C. sapidus* may be influenced profoundly by such offshore recruitment.

This work is part of a broader program of research on crab larval behavior supported by the Maryland Sea Grant program (Project No. R/F-8). We wish to thank Mr. Robert Miller and Mr. Robert McConnaughey for their technical assistance.

SUMMARY

1. The first zoeal stage of *Callinectes sapidus* shows negative geotaxis unaffected by salinity changes of 5‰; high barokinesis at pressure increments above 1 atm; an increase in swimming rate with a salinity increase; and maintenance of swimming rate as temperature drops.

2. Stage IV larvae show both positive and negative geotaxis. As salinity drops, positive geotaxis prevails; as it increases negative geotaxis prevails. Stage IV larvae show a tendency to reduce swimming rate as pressure increases, as temperature drops, and as they become acclimated to higher salinities.

3. Stage VII larvae show positive geotaxis and reduced swimming rate in response to increased pressure, reduced temperature, and as they are acclimated to increased salinity.

4. Between hatching and the seventh (terminal) zoeal stage, passive sinking rate increases 3.2-fold, while swimming rate increases 4.4-fold.

5. These responses to environmental stimuli produce a pattern of early stages moving to surface waters and later stages to deeper waters.

6. Because of characteristic circulation in lower estuarine and coastal marine systems, this pattern of vertical distribution could provide a mechanism for exchange of larvae between the estuary and the coastal marine environment.

7. In stratified estuaries, offshore recruitment may significantly influence population dynamics in *C. sapidus*.

LITERATURE CITED

BEARDSLEY, R. C., W. C. BOICOURT, AND D. V. HANSEN, 1976. Physical oceanography of the Mid-Atlantic Bight. American Society of Limnology and Oceanography, Special *Symposium*, **2**: 20–34.

BENTLEY, E., AND S. D. SULKIN, 1977. The ontogeny of barokinesis during the zoeal development of the xanthid crab *Rhithropanopeus harrisii* (Gould). *Mar. Behav. Physiol.*, **4**: 275–282.

BOUSFIELD, E. L., 1955. Ecological control of the occurrence of barnacles in the Miramichi Estuary. *Nat. Mus. Can. Bull.*, **137**: 1–69.

BUMPUS, D. F., 1965. Residual drift along the bottom on the continental shelf in the middle Atlantic Bight area. *Limnol. Oceanogr.*, **10** (Supplement): R50–53.

CARRIKER, M. R., 1951. Ecological observations on the distribution of oyster larvae in New Jersey estuaries. *Ecol. Monogr.*, **21**: 19–38.

DUDLEY, D. L., AND M. H. JUDY, 1971. Occurrence of larval, juvenile, and mature crabs in the vicinity of Beaufort Inlet, North Carolina. *NOAA Tech. Report*, NMFS, *Special Scientific Report, Fisheries*, **637**: 1–10.

FORWARD, R. B., JR., AND J. D. COSTLOW, JR., 1974. The ontogeny of phototaxis by larvae of the crab *Rhithropanopeus harrisii*. *Mar. Biol.*, **25**: 27–33.

GOY, J. W., 1976. *Seasonal distribution and retention of some decapod crustacean larvae within the Chesapeake Bay, Virginia.* Masters thesis, Old Dominion University. 334 pp.

HARDY, A. C., AND R. BAINBRIDGE, 1951. Effects of pressure on the behavior of decapod larvae. *Nature*, **167**: 354–355.

HARRISON, W., J. J. NORCROSS, N. A. PORE, AND E. M. STANLEY, 1967. Circulation of shelf waters off Chesapeake Bight—surface and bottom drift of continental shelf waters between Cape Henlopen, Delaware and Cape Hatteras, North Carolina. June 1963–December 1964. *United States ESSA Professional Papers*, **3**: 1–82.

HOPKINS, S. H., 1943. The external morphology of the first and second zoeal stages of the blue crab, *Callinectes sapidus* Rathbun. *Biol. Bull.*, **87**: 145–152.

LATZ, M. I., AND R. B. FORWARD, JR., 1977. The effect of salinity upon phototaxis and geotaxis in a larval crustacean. *Biol. Bull.*, **153**: 163–179.

MILEIKOVSKY, S. A., 1972. The "pelagic larvaton" and its role in the biology of the World Ocean, with special reference to pelagic larvae of marine bottom invertebrates. *Mar. Biol.*, **16**: 13–21.

NAYLOR, E., AND M. J. ISAAC, 1973. Behavioral significance of pressure responses in megalopa larvae of *Callinectes sapidus* and *Macropipus* sp. *Mar. Behav. Physiol.*, **1**: 341–350.

NICHOLS, P., AND P. M. KENEY, 1963. Crab larvae (*Callinectes*) in plankton collections from cruises of M/V Theodore N. Gill. South Atlantic coast of the United States, 1953–1954. U. S. Fish and Wild. Serv. *Spec. Sci. Rep. Fish.*, **448**: 1–14.

OTT, F. S., AND R. B. FORWARD, JR., 1976. The effect of temperature on phototaxis and geotaxis by larvae of the crab *Rhithropanopeus harrisii* (Gould). *J. Exp. Mar. Biol. Ecol.*, **23**: 97–107.

PRITCHARD, D. W., 1952. Salinity distribution and circulation in the Chesapeake Bay estuarine system. *J. Mar. Res.*, **11**: 106–123.

RICE, A. L., 1964. Observations on the effects of changes in hydrostatic pressure on the behavior of some marine animals. *J. Mar. Biol. Assn. U. K.*, **44**: 163–175.

RICE, A. L., 1966. The orientation of pressure responses of some marine crustacea. Proceedings of Symposium on Crustacea, Part III, *Mar. Biol. Assn., India,* **1966**: 1124–1131.

SANDIFER, P. A., 1973. Distribution and abundance of decapod crustacean larvae in the York River Estuary and adjacent lower Chesapeake Bay, Virginia, 1968–1969. *Chesapeake Sci.*, **14**: 235–257.

SANDIFER, P. A., 1975. The role of pelagic larvae in recruitment to populations of decapod crustaceans in the York River Estuary and adjacent lower Chesapeake Bay, Virginia. *Estuarine Coastal Mar. Sci.*, **3**: 269–279.

SCHELTEMA, R. S., 1975. Relationship of larval dispersal, gene-flow, and natural selection to geographic variations of benthic invertebrates in estuaries and along coastal regions. *Estuarine Research*, **1**: 372–391.

SULKIN, S. D., 1973. Depth regulation of crab larvae in the absence of light. *J. Exp. Mar. Biol. Ecol.*, **13**: 73–82.

SULKIN, S. D., 1975. The influence of light in the depth regulation of crab larvae. *Biol. Bull.*, **148**: 333–343.

SULKIN, S. D., 1978. Nutritional requirements during larval development of the portunid crab, *Callinectes sapidus* Rathbun. *J. Exp. Mar. Biol. Ecol.*, **34**: 29–41.

SULKIN, S. D., I. PHILLIPS, AND W. VAN HEUKELEM, 1979. On the locomotory rhythm of brachyuran crab larvae and its significance in vertical migration. *Marine Ecology—Progress Series*, **1**: 331–335.

THORSON, G., 1950. Reproductive and larval ecology of marine bottom invertebrates. *Biol. Rev. of Camb. Philos. Soc.*, **25**: 1–45.

VAN ENGEL, W. A., 1958. The blue crab and its fishery in Chesapeake Bay. I. Reproduction, early development, growth, and migration. *Comm. Fish. Rev.*, **20**: [illegible]–17.

WHEELER, D., AND C. E. EPIFANIO, 1978. Behavioral response to hydrostatic pressure in larvae of two species of xanthid crabs. *Mar. Biol.*, **46**: 167–174.

WOOD, L., AND W. J. HARGIS, 1971. Transport of bivalve larvae in a tidal estuary. Pp. 29–44 in D. J. Crisp, Ed., *Fourth European Marine Biology Symposium*, Cambridge Univ. Press, Cambridge, England.

Reference: *Biol. Bull.*, **159**: 418–427. (October, 1980)

ELECTROPHORETIC VARIATION IN SYMPATRIC MUD CRABS FROM NORTH INLET, SOUTH CAROLINA

KATHERINE TURNER AND TIMOTHY A. LYERLA

Department of Biology, Clark University, Worcester, Massachusetts 01610

In the decapod crustaceans, electrophoretic screening of enzymes and proteins has shown that this group, in general, possesses low levels of detectable genetic variation (Hedgecock *et al.*, 1976; Gooch, 1977; Nemeth and Tracey, 1979). Gooch (1977) has hypothesized that this may be a characteristic of the decapods, and Nemeth and Tracey (1979) suggest it may be due to low rates of mutation or of intracistronic recombination. Since both evolutionary (Lewontin, 1974) and ecological (Nevo, 1978) inferences are made from genetic variation in organisms measured with this technique, it is important that the generalization of low variability in decapod crustaceans be thoroughly tested by sampling several different members of this group under diverse ecological conditions.

To this end, we have assessed genetic variation in xanthid mud crab populations at North Inlet, South Carolina, using gel electrophoresis. These studies provide three additional populations to add to the data concerning electrophoretic variability in the decapods, and deal with genera that have been studied previously as to their ecological relationships (McDonald, 1977) and a species in which taxonomic "forms" have been described (Rathbun, 1930).

At North Inlet, both *Panopeus herbstii* and *Eurypanopeus depressus,* which are frequently associated with oyster bar communities along the eastern Atlantic seaboard of the United States (Lunz, 1937; Ryan, 1956; Williams, 1965), coexist in the mud and crevices between and under shells on intertidal oyster bars. *P. herbstii* is the larger of the two in adult stages but overlaps sizes of *E. depressus* during juvenile stages. Trophic differences between juveniles appear to be a major mechanism of niche partitioning that permits their successful coexistence (McDonald, 1977). In addition, morphological varieties or "forms" of *P. herbstii* occur at this site, including the forms "simpsoni" and "obesa," among the four types described within the geographic range of this species (Rathbun, 1930). The form "typica" also occurs at North Inlet but was not found in sufficient numbers for this study.

MATERIALS AND METHODS

Collection

Adult specimens of *E. depressus* and the two forms of *P. herbstii* were collected from October, 1977, through October, 1978, from Town Creek at North Inlet, South Carolina. Data for this study were derived from summer (July) and fall (October) collections of 1978. Animals collected during the fall (October) and winter (December) of 1977 and spring (March) of 1978 were used primarily to assess techniques. Data from these collections indicated no overt seasonal differences. Although *E. depressus* was found throughout the intertidal region, the two forms of *P. herbstii* occurred in generally discrete but partly overlapping areas:

"obesa" in the upper intertidal region where the marsh grass, *Spartina alterniflora*, was abundant, and "simpsoni" in the lower areas where oyster rubble and clumps of the oyster *Crassostrea virginica* covered the surface. In the mid-intertidal, which was characterized by a relatively flat mud surface, both forms could be found, although "obesa" appeared to be more abundant. Crabs were identified by morphological characteristics outlined by Rathbun (1930) and McDonald (1977).

Electrophoresis

As these crabs were so small, *E. depressus* homogenates were prepared from whole animals. The appendages, dorsal carapace and hepatopancreas were removed from *P. herbstii* before samples were homogenized. Otherwise, the homogenization procedure was the same for both genera. The homogenizing buffer was comprised of 10 mM Tris and 10 mM maleic acid, pH 7.4, with 10 mM disodium ethylene diaminetetraacetic acid (EDTA), 10 mM $MgCl_2$, 0.4 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 0.1% (v/v) 2-mercaptoethanol and 10% (v/v) 1° octanol added. Homogenates (40% w/v) were prepared in an ice bath with Polytron PCU-2 and then centrifuged for 20 min at 900 × *g* in a Beckman Microfuge B in a cold room at 4°C.

Soluble and particulate fractions were separated in some samples in order to evaluate the banding patterns of enzymes. For these separations, homogenates were prepared according to the methods of Lyerla *et al.* (1979), using 0.3 M sucrose in 10 mM Tris/citrate buffer, pH 7.4, with 10% (v/v) 1° octanol added to reduce foaming. Supernatants were saved and final pellets dispersed in the regular homogenizing buffer to be analyzed electrophoretically as the soluble and particulate fractions, respectively.

Samples were screened on 13% (w/v) Connaught starch gels using standard horizontal electrophoresis techniques for all systems except amylase. Electrophoretic buffer systems included the following:

A) 0.1 M Tris/maleate (Shaw and Prasad, 1970), B) 0.41 M Sodium citrate/citrate (Brewer, 1970), C) 0.155 M Tris/citrate (Shaw and Prasad, 1970), D) 0.5 M Tris/disodium EDTA/borate (Shaw and Prasad, 1970), and E) 0.05 M K_2HPO_4/KH_2PO_4, pH 7.0, with gel buffer a 1 : 15 dilution of electrode buffer.

Electrophoresis was 70–95 V for 16 hr using buffer systems A, C, D, and E, and for 4 hr with buffer system B. All runs were standardized with bromophenol blue tracking dye, which migrated 8 cm from the origin. Staining recipes are given below and are modifications of standard methods (Shaw and Prasad, 1970). Dyes, substrates, co-factors and auxiliary enzymes were purchased from Sigma Chemical Co. Unless otherwise noted, gels were incubated at 37°C until well defined bands were present (usually within 1–2 hr).

Staining recipes were: *Aldolase* (ALD): 275 mg fructose-1, 6-diphosphate (trisodium salt), 100 U glyceraldehyde-3-phosphate dehydrogenase, 75 mg sodium arsenate, 25 mg nicotinamide adenine dinucleotide (NAD^+), 15 mg nitroblue tetrazolium (NBT), 2 mg phenazine methosulfate (PMS) in 50 ml of 0.4 M Tris/HCl buffer, pH 8.0. *Alkaline phosphatase* (AKPH): 50 mg sodium α-naphthyl acid phosphate, 50 mg fast blue BB, 30 mg $MgCl_2$, 30 mg $MnCl_2$, 1 g NaCl in 50 ml of 0.04 M Tris/HCl buffer, pH 8.0. *Beta-glucuronidase* (b-GLU): 20 mg 4-methylumbelliferyl-β-D-glucuronide in 5 ml of 0.05 M Na_2HPO_4/citric acid buffer, pH 4.0. Gel surface flooded and incubated at 37°C for 45 min. Rinsed once with H_2O and 7.4 N NH_4OH added dropwise to cover surface of the gel. Enzyme activity marked by bands of fluorescence when gel viewed immediately under UV illumination. *Catalase* (CAT): 0.0015% H_2O_2 (v/v) in 0.01 M NaH_2PO_4/NaOH buffer, pH 7.0. Gel soaked for 15 min at room temperature, rinsed once with H_2O and stained with 0.09 M KI. White bands against dark blue background appear after 20 min at room temperature, indicating sites of enzyme activity. *Esterase* (EST): Gel soaked for 30 min in 0.05 M boric acid at 4°C before staining. 30 mg α-naphthyl acetate, 30 mg α-naphthyl butyrate, 15 mg α-naphthyl proprionate, 100 mg fast blue RR in 50 ml of 0.05 M Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0. *Glutamate-oxaloacetate transaminase* (GOT): 1 mg pyridoxal-5′-phosphate, 200 mg L-aspartic acid, 100 mg α-ketoglutaric acid, 150 mg fast blue BB in 50 ml of 0.2 M Tris/HCl buffer, pH 8.0. *Glucose-6-phosphate dehydrogenase* (G6PD): 200 mg glucose-6-phosphate (disodium salt), 50 mg $MgCl_2$, 15 mg $NADP^+$, 15 mg NBT, 2 mg PMS in 50 ml of 0.04 M Tris/HCl buffer, pH 8.0. *Glutamate-pyruvate transaminase* (GPT): 30 mg NADH, 50 mg L-alanine, 20 mg α-ketoglutaric acid, 300 U lactate dehydrogenase (Type III) in 10 ml of 0.02 M Tris/HCl buffer, pH 8.0. Gel surface flooded wth 5 ml stain and incubated at 37°C until non-fluorescent sites (enzyme activity) in fluorescent gel surface appeared under UV illumination. *Isocitrate dehydrodehydrogenase* (IDH): 200 mg isocitrate (trisodium salt), 50 mg $MgCl_2$, 15 mg $NADP^+$, 15 mg NBT, 2 mg PMS in 50 ml of 0.04 M Tris/HCl buffer, pH 8.0. *Malate dehydrogenase* (MDH): 600 mg L-malate (monosodium salt), 25 mg NAD^+, 15 mg NBT, 2 mg PMS in 50 ml of 0.04 M Tris/HCl buffer, pH 8.0. *Malic enzyme* (ME): 600 mg L-malate, 50 mg $MgCl_2$, 15 mg $NADP^+$, 15 mg NBT, 2 mg PMS in 50 ml of 0.04 M Tris/HCl buffer, pH 8.0. *6-phosphogluconate dehydrogenase* (6-PGD): 100 mg 6-phosphogluconic acid, 50 mg $MgCl_2$, 15 mg $NADP^+$, 15 mg NBT, 2 mg PMS in 50 ml of 0.04 M Tris/HCl buffer, pH 8.0. *Phosphoglucose isomerase* (PGI): 80 mg fructose-6-phosphate (disodium salt), 50 mg $MgCl_2$, 80 U glucose-6-phosphate dehydrogenase (Type XI), 7.5 mg $NADP^+$, 10 mg MTT, 2 mg PMS in 50 ml of 0.04 M Tris/HCl buffer, pH 8.0. *Phosphoglucomutase* (PGM): 300 mg glucose-1-phosphate (disodium salt), 80 U glucose-6-phosphate dehydrogenase, 50 mg $MgCl_2$, 15 mg $NADP^+$, 20 mg MTT, 2 mg PMS in 50 ml of 0.04 M Tris/HCl buffer, pH 7.0. *Protein* (PRO): 200 mg nigrosin in 25 ml 95% methanol, 25 ml H_2O, 5 ml glacial acetic acid. Gel stained for 10 min at 37°C and destained with several washes of methanol/acetate acid fixative without nigrosin over 1–2 days at room temperature. *Tetrazolium oxidase* (TO): 25 mg NAD^+, 15 mg NBT, 2 mg PMS in 50 ml of 0.04 M Tris/HCl buffer, pH 8.0.

Amylase activity was visualized by electrophoresis on horizontal polyacrylamide gel slabs [6% acrylamide (w/v)] followed by incubation of the acrylamide gel with an underlying gel of 2 parts agar (Difco Bactoagar) and 1 part potato starch (Sigma Chemical Co.) with 3.4 mM NaCl in gel buffer for 90 min at 37°C (modified from Evans *et al.*, 1977). Buffer system C was used for both gels. After incubation, the acrylamide gel was discarded, and the agar gel stained with a 0.3% (w/v) KI, 0.15% (w/v) I_2 solution with amylase activity appearing as translucent bands against a dark blue background.

Putative allelic variants of polymorphic loci were identified by the distance, in mm, of their migration from the origin to the center of the band of activity. Only those bands differing by 1.5 mm or greater were considered as allelic variants resolved in these systems.

TABLE I

Summary of electrophoretically scorable loci for three populations of xanthid mud crabs (P. herbstii *form "simpsoni,"* P. herbstii *form "obesa" and* E. depressus).

Enzyme/protein	Abbreviation/ no. loci	Buffer system†	Variation††	Populations [illegible]
1. Amylase	AMY/1	C	P	All three
2. Catalase	CAT/1	B	M	All three
3. Beta-glucuronidase	b-GLU/1	B	M	All three
4. Glutamate-oxaloacetate transaminase	GOT/1	C, E	M	All three
5. Glutamate-pyruvate transaminase	GPT/1	A	M	All three
6. Glucose-6-phosphate dehydrogenase	G6PD/1	E	M	All three
7. 6-phosphogluconate dehydrogenase	6PGD/1	A	M	All three
8. Tetrazolium oxidase	TO/1	D	M	All three
9. Alkaline phosphatase	AKPH-1	A, E	M	*E. depressus*
	AKPH-2		M	*E. depressus*
10. Aldolase	ALD-1	B	M	All three
	ALD-2		M	All three
11. Isocitrate dehydrogenase	IDH-1	B	M	*P. herbstii*
	IDH-2		M	All three
12. Malate dehydrogenase	MDH-1	A, C	M/P*	All three
	MDH-2		M/P**	All three
13. Malic enzyme	ME-1	D	M	All three
	ME-2		M	All three
14. Phosphoglucose isomerase	PGI-1	D	M	All three
	PGI-2		M	All three
15. Phosphoglucomutase	PGM-1	C	M	All three
	PGM-2		M	All three
16. Esterase	EST-1	D	M	form "simpsoni"
	EST-2		M	*E. depressus*
	EST-3		M	*E. depressus*
	EST-4		M	All three
17. Protein	PRO-1	C	M	All three
	PRO-2		M	All three
	PRO-3		M	All three

* In form "simpsoni" only.
** In form "simpsoni" and *E. depressus*.
† See Materials and Methods.
†† P = polymorphic; M = monomorphic.

RESULTS

Sixteen different enzyme systems, as well as soluble proteins, were screened in both genera (Table I). These gave a total of 27 scorable loci in *E. depressus*, 25 in *P. herbstii* "simpsoni" and 24 in *P. herbstii* "obesa." Eight enzymes exhibited a single band of activity, and were considered as products of a single structural gene in each population. Seven enzyme systems appeared as double bands from whole, unfractionated homogenates. From fractionation studies, it was determined that the double banded condition was due to the presence of both soluble and particulate forms, and therefore the two bands were considered products from two structural genes. The anodal-most band was designated "-1" and the other "-2". Esterase (EST) and soluble protein (PRO) exhibited multiple bands.

TABLE II

Allele frequencies at polymorphic loci in E. depressus (*a*), P. herbstii *form "obesa"* (*b*) *and* P. herbstii *form "simpsoni"* (*c*).

Loci	Sample size*			Allele**	Allele frequency		
	a	b	c		a	b	c
Amylase	54	20	79	125	—	0.300	—
				116	—	0.100	—
				113	—	0.200	0.158
				109	—	0.150	0.392
				105	—	0.125	0.210
				103	—	0.125	0.240
				100	0.676	—	—
				95	0.324	—	—
				Hobs	0.278***	0.600***	0.468***
				Hexp	0.442	0.826	0.724
MDH-1	45	20	89	120	—	1.000	0.950
				111	—	—	0.050
				100	1.000	—	—
				Hobs	—	—	0.101
				Hexp	—	—	0.096
MDH-2	51	16	109	100	0.931	1.000	0.985
				94	—	—	0.015
				92	0.069	—	—
				Hobs	0.096	—	0.029
				Hexp	0.126	—	0.029

* Sample size represents the number of individuals screened at each locus.

** Alleles were numbered by assigning "100" to the most common allele at each locus in *E. depressus* and adding or substracting the difference in mobility (in mm) for other alleles.

*** Observed and expected heterozygosities were significantly different from the expectations of Hardy-Weinberg equilibrium ($P < 0.05$).

P. herbstii "simpsoni" had the greatest number of esterase bands, but only two were consistently resolved: EST-1, which was unique to "simpsoni," and EST-4, which co-migrated with an esterase zone found in both *P. herbstii* "obesa" and *E. depressus*. Esterase zymogram patterns in *P. herbstii* "obesa" were clearly distinct from those of *P. herbstii* "simpsoni" or *E. depressus*, but only one zone of activity, the common EST-4 site, was consistently resolved. The esterase pattern from *E. depressus* was also readily distinguished from those of both forms of *P. herbstii* and had two zones unique to this genus, EST-2 and EST-3, as well as the third, common site, EST-4. Thus, four electrophoretically separable zones of esterase activity found among the three populations could be resolved in all samples and are presumably coded for by separate structural genes. Electrophoretic variations that could be ascribed to presumptive allelic variants for these sites were not found, and these genes are scored as monomorphic (Table I). Three bands of soluble protein were consistently resolved in all samples. The arrays from both forms of *P. herbstii* were identical. In *E. depressus*, the two anodal-most bands were faster migrating than those in *P. herbstii*, whereas the third site co-migrated with that found in this genus. As in the esterase zones of activity, no putative allelic variants of the three sites of soluble protein were observed. These were scored as the products of three different structural genes, PRO-1, -2 and -3, which were monomorphic in each population (Table I).

Nineteen monomorphic loci were scorable in all three populations (Table I). *E. depressus* was also monomorphic at AKPH-1 and -2, EST-2 and -3, and MDH-1, while *P. herbstii* "simpsoni" was monomorphic at EST-1 and -4 and IDH-1 and *P. herbstii* "obesa" at EST-4, IDH-1, and MDH-1 and -2. Although *E. depressus* and both forms of *P. herbstii* were monomorphic at the same 19 loci, at only three of these, EST-4, GOT and PRO-3, did the three populations share electromorphs. The remaining 16 loci were fixed for electrophoretically different sites in the two genera. The "100" allele at MDH-2 was also common to both genera.

Allelic frequencies at polymorphic loci are presented in Table II. A locus was classified as polymorphic if the frequency of the most common allele was less than 0.99 (Nei, 1975). The expected heterozygosity at each locus was calculated using Levene's (1949) formula for finite samples and was compared to the observed heterozygosity to determine if there was a significant deviation ($P < 0.05$) from the expectations of Hardy-Weinberg equilibrium (Table II). Disequilibrium was observed for AMY, but not MDH, in all three populations.

Values for the parameters P and $\bar{H}$ for each population are given in Table III. Using a *t*-test for comparisons (Crow and Kimura, 1970), the observed and expected $\bar{H}$ values within each population are not significantly different, and neither the observed $\bar{H}$ values nor the expected $\bar{H}$ values between populations differ at the 0.05 level of significance.

A difference between the two forms of *P. herbstii* was observed at the AMY locus, with six allozymes present in the "obesa" form and four in "simpsoni" (Table II; Fig. 1). The four allozymes in the "simpsoni" population were shared by "obesa," but at lower frequencies in "obesa." Other possible genetic differences between the two forms are at the two MDH loci. Both were polymorphic in "simpsoni" and appeared to be monomorphic in "obesa." However, due to the small sample size of "obesa" ($N = 20$ at MDH-1; $N = 16$ at MDH-2) and the low frequency of heterozygotes at these loci in "simpsoni" ($H = 0.101$ at MDH-1; $H = 0.029$ at MDH-2), it is possible that "obesa" is polymorphic at these loci. That is, the probability of not having sampled a heterozygote in "obesa" if the frequencies of homozygotes at these loci are the same in each population is greater than 0.05 [$(0.899)^{20} = 0.12$ at MDH-1; $(0.971)^{16} = 0.62$ at MDH-2].

DISCUSSION

In this study, the classification of a particular *P. herbstii* crab as a "simpsoni" or an "obesa" form variant by morphological characters was supported by amylase

TABLE III

Proportion of polymorphic loci (P) and mean heterozygosities ($\bar{H}$) in E. depressus, P. herbstii *form "obesa" and* P. herbstii *form "simpsoni." $\bar{H}obs$ = observed and $\bar{H}exp$ = expected heterozygosities. SE = standard error.*

Population	Loci	P	$\bar{H}$obs ± SE	$\bar{H}$exp ± SE
E. depressus	27	0.074	0.015 ± 0.0004	0.021 ± 0.0012
P. herbstii form "obesa"	24	0.042	0.025 ± 0.0025	0.034 ± 0.0049
P. herbstii form "simpsoni"	25	0.120	0.024 ± 0.0006	0.034 ± 0.0017

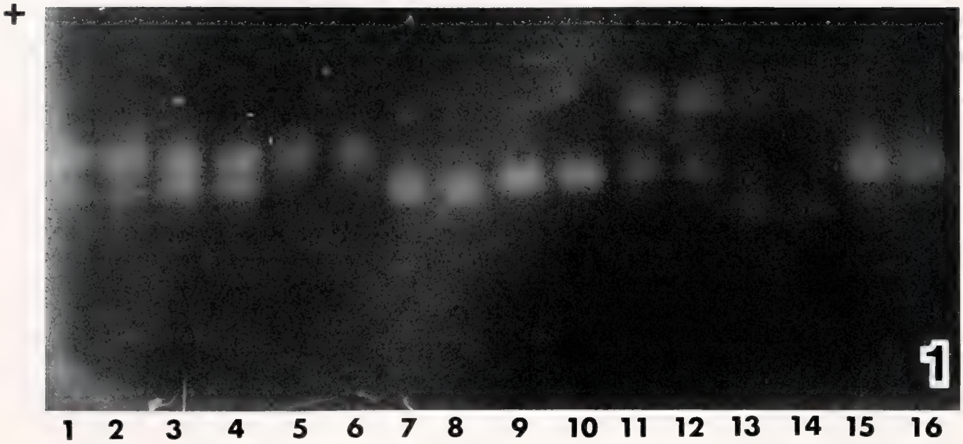


FIGURE 1. Polyacrylamide gel zymogram of amylase activity in *Panopeus herbstii.* Channels 1–10 are duplicate samples from five different *P. herbstii* form "simpsoni" individuals, and channels 11–16 are duplicates from three different *P. herbstii* form "obesa" individuals. Their inferred genotypes are as follows: channels 1–2 and 3–4, 109/105; channels 5–6, 113/113; channels 7–8, 103/103; channels 9–10, 105/105; channels 11–12, 125/105; channels 13–14, 125/103; channels 15–16, 105/105. At most, only two bands of amylase activity were resolved from a given individual, indicating a monomeric protein with electrophoretically separable allelic variants.

and/or esterase zymogram patterns. Using Nei's index (Nei, 1975) to estimate genetic similarity, however, the two forms are genetically identical ($I = 0.997 \pm 0.011$ SE, where I ranges from 1, or total identity, to 0, with no shared alleles in two populations). This is due to the large number of monomorphic loci at which the two forms shared electromorphs (20 out of 23). On an individual gene basis, those contributing to an identity of less than 1 were polymorphic: MDH-1 and AMY. Yet the two forms' dissimilar esterase zymogram patterns and the presence at the AMY locus of two alleles in "obesa" not found in "simpsoni" may indicate a greater genetic difference between these two populations than that implied by their designation as form variants (Rathbun, 1930). Differences between two populations of loci such as those coding for non-specific esterases may mean these could be considered as genetically divergent populations (Sarich, 1977).

Although the two genera, *P. herbstii* and *E. depressus,* were readily distinguished at most loci, they shared electromorphs at EST-4, GOT, PRO-3 and MDH-2. Other similarities were also found. The scorable polymorphisms occurred only at the AMY and MDH loci, and the frequency of heterozygotes was low for MDH and high for AMY in both genera. Also, allele frequencies at the MDH loci in both genera conformed to the expectation of Hardy-Weinburg equilibrium but not at AMY in either genera.

These results provide some insights into current problems of observed genetic variation in decapod crustaceans. The electromorphic differences of some 19 out of 23 scorable loci between *P. herbstii* and *E. depressus* indicate there has been sufficient evolutionary time (and a high enough mutation rate) to allow a genetic distinction, even at the single gene level, between these two sympatric genera. Their ecological niches may overlap considerably (McDonald, 1977), but this obviously does not require overlap of electromorphic forms of structural genes. Also, genetic variation within each genus, as measured by mean heterozygosities, is low and similar to estimates for other decapod crustaceans (Gooch and Schopf, 1972; Hedgecock *et al.*, 1976; Gooch, 1977; Cole and Morgan, 1978; Costa and Bisol, 1978). This estimate is made without respect to the types of genes contributing to it. Yet *P. herbstii* and *E. depressus* are polymorphic at the same two loci, AMY and MDH, and monomorphic at more than 19 other scorable genes. MDH has usually been included in studies of genetic variability in other decapod species, and there does not appear to be a tendency for these genes to be more frequently polymorphic than others (Gooch and Schopf, 1972; Tracey *et al.*, 1975; Gooch, 1977; Cole and Morgan, 1978; Costa and Bisol, 1978; Nemeth and Tracey, 1979). In addition to this study, electrophoretic variation of AMY has apparently been reported in other decapod crustaceans only by Nemeth and Tracey (1979) in their survey of crayfish populations (*Orconectes* spp. and *Cambarus* spp.). The locus was monomorphic in all but one population. It seems reasonable to assume, then, that the specificity of the observed polymorphisms in *P. herbstii* and *E. depressus* is of biological importance to both genera. The disequilibrium at the AMY locus and the low likelihood of having the same two genes and only these two, showing allelic variation in two sympatric genera suggest that selective influences rather than random changes account for the nature of this variation.

Finally, the low values of $\bar{H}$ in all three populations support the suggestion that low genetic variability is a phylogenetic character of the decapod crustaceans (Gooch, 1977). Gooch (1977) examined three separate populations and three different stages of development for electromorphic variation of some 15 loci in the xanthid crab, *Rithropanopeus harrisii* and found no life-cycle or geographic variability, with the possible exception of a polymorphic peptidase locus in a Maryland population. As Gooch (1977) points out, these estimates of low genetic variability in decapods, expressed as the low incidence of electrophoretic variability at protein loci, are likely to remain valid even if a larger number of loci were screened. In this study, where the number of monomorphic loci is biased upward by including only those esterase bands that exhibited no genetic variation, refinement of techniques or parent-offspring data that might provide possible allelic variants in other, poorly resolved zones would not appreciably alter the overall heterozygosity estimates for these genera. Nemeth and Tracey (1979) have suggested that low mutation or recombination rates may explain the low variability in this taxonomic group. However, the genetic distinctions between these two sympatric genera, *P. herbstii* and *E. depressus*, are compatible with average mutation rates expected to account for the accumulation of structural gene differences between two genera at selectively neutral genes (Sarich, 1977). The specificity of their polymorphic loci imply a selective advantage for some of the allelic variants of AMY and MDH, and it is here that further work with genetic variation in these two genera would seem most appropriate.

We are grateful to Dr. Jack McDonald, Smithsonian Institution, Fort Pierce Bureau, Fort Pierce, Florida, for providing much valuable information and to Dr. Alan M. Young, Nasson College, Springvale, Maine, for his assistance in collecting animals; for the use of the facilities at the Belle W. Baruch Institute for Marine Biology and Coastal Research, University of South Carolina; and to Dennis Allen and the staff at the Baruch Institute for their assistance and hospitality. This research was supported in part by funds from the Lerner Fund for Marine

Biology of the American Museum of Natural History and by a Grant-in-Aid for Research from Sigma Xi to Katherine Turner. It was completed in partial fulfillment of the requirements for the Master of Arts degree in Biology from Clark University to Katherine Turner.

SUMMARY

The genetic variation of 27 scorable genes in *Eurypanopeus depressus,* 25 in *Panopeus herbstii* form "simpsoni" and 24 in *P. herbstii* form "obesa" at Town Creek, North Inlet, South Carolina, was studied by gel electrophoresis. The three populations had low levels of genetic variability, comparable to those found in other decapod crustaceans, with polymorphisms observed only at the amylase and malate dehydrogenase loci. The two forms of *P. herbstii* could be distinguished both by previously described morphological differences and by genetic differences found in this study. The amounts of genetic variation in *E. depressus* and *P. herbstii* were similar, as measured by $\overline{H}$, but shared electromorphs at only 4 of 23 scorable loci. The common, but specific, polymorphisms at amylase and malate dehydrogenase were taken to imply selective influences as a factor in their maintenance within these two sympatric genera.

LITERATURE CITED

BREWER, G. J., 1970. *An Introduction to Isozyme Techniques.* Academic Press, New York, 186 pp.

COLE, M. A., AND R. P. MORGAN, 1978. Genetic variation in two populations of blue crab, *Callinectes sapidus. Estuaries,* **1**: 202–204.

COSTA, R., AND P. M. BISOL, 1978. Genetic variability in deep sea organisms. *Biol. Bull.,* **155**: 125–133.

CROW, J. F., AND M. KIMURA, 1970. *An Introduction to Population Genetics Theory.* Harper and Row, New York, 591 pp.

EVANS, M. J., L. L. HUANG, AND W. D. DAWSON, 1977. Genetic variation of amylases in deer mice. *J. Hered.,* **68**: 313–316.

GOOCH, J. L., 1977. Allozyme genetics of life cycle stages of brachyurans. *Chesapeake Sci.,* **18**: 284–289.

GOOCH, J. L., AND T. J. M. SCHOPF, 1972. Genetic variability in the deep sea: relation to environmental variability. *Evolution,* **26**: 545–552.

HEDGECOCK, D., R. A. SHLESER, AND K. NELSON, 1976. Applications of biochemical genetics to aquaculture. *J. Fish. Res. Bd., Can.,* **33**: 1108–1119.

LEVENE, H., 1949. On a matching problem arising in genetics. *Ann. Math. Stat.,* **20**: 91–94.

LEWONTIN, R. C., 1974. *The Genetic Basis of Evolutionary Change.* Columbia University Press, New York, 346 pp.

LUNZ, G., 1937. Xanthidae (mud crabs) of the Carolinas. *Charleston Museum Leaflet* No. **9**: 9–27.

LYERLA, J. H., T. A. LYERLA, AND S. E. JOHNSON, 1979. Malate dehydrogenase isozyme patterns in species of terrestrial isopods. *Comp. Biochem. Physiol.,* **63**: 19–24.

MCDONALD, H. J., 1977. *The comparative intertidal ecology and niche relations of the sympatric mud crabs* Panopeus herbstii *Milne-Edwards and* Eurypanopeus depressus *(Smith) at North Inlet, South Carolina, U. S. A. (Decapoda: Brachyura: Xanthidae).* Ph.D. Thesis, University of South Carolina, 233 pp., DAI 38/098, p. 4109.

NEI, M., 1975. *Molecular Population Genetics and Evolution.* American Elsevier Publ. Co., Inc., New York, 288 pp.

NEMETH, S. T., AND M. L. TRACEY, 1979. Allozyme variability and relatedness in six crayfish species. *J. Hered.,* **70**: 37–43.

NEVO, E., 1978. Genetic variation in natural populations: patterns and theory. *Theor. Popul. Biol.,* **13**: 121–177.

RATHBUN, M., 1930. The cancroid crabs of North America of the families Euryalidae, Portunidae, Atelecyclidae, Cancridae, and Xanthidae. *U. S. Nat. Mus. Bull.*, **152**: 1–609.

RYAN, E., 1956. Observations on the life histories and the distribution of the Xanthidae of Chesapeake Bay. *Am. Nat.*, **56**: 138–162.

SARICH, V. M., 1977. Rates, sample sizes, and the neutrality hypothesis in evolutionary studies. *Nature*, **256**: 24–28.

SHAW, C. R., AND R. PRASAD, 1970. Starch gel electrophoresis—a compilation of recipes. *Biochem. Genet.*, **5**: 297–320.

TRACEY, M. L., J. NELSON, D. HEDGECOCK, R. A. SHLESER, AND M. L. PRESSICK, 1975. Biochemical genetics of lobsters: genetic variation and the structure of the American lobster (*Homarus americanus*) populations. *J. Fish. Res. Bd., Can.*, **32**: 2091–2101.

WILLIAMS, A., 1965. Marine decapod crustaceans of the Carolinas. *Fishery Bulletin* [U. S. Fish and Wildlife Service], **65**: 1–298.

Reference: *Biol. Bull.*, **159**: 428–440. (October, 1980)

ORIENTATION AND CURRENT-INDUCED FLOW IN THE STALKED ASCIDIAN *STYELA MONTEREYENSIS*

CRAIG M. YOUNG AND LEE F. BRAITHWAITE

Department of Zoology, University of Alberta, Edmonton, Alberta T6G 2E9, Canada; and Department of Zoology, Brigham Young University, Provo, Utah 84602

Virtually all sessile suspension feeders rely on ambient water movement to renew their food supplies and to carry away waste products and depleted water. In addition, species in many taxa have developed hydrodynamic mechanisms for exploiting the energy in the velocity gradient between the substratum and the moving water. For instance, exogenous currents induce flow through tubes, burrows, or internal chambers, and across external food-collecting surfaces (Vogel, 1978). Where currents are predictably unidirectional or bidirectional, colonial organisms frequently exhibit a permanent orientation which maximizes exposure to current (Wainwright and Dillon, 1969; Grigg, 1972; Meyer, 1973). In areas with turbulent water or currents of unpredictable direction, orientation-independent mechanisms are common (Warner, 1977). These are exemplified by animals with irregular or radial forms, such as sponges (Vogel, 1974) and some crinoids (Meyer, 1973). While barnacles (Crisp and Stubbings, 1957) and brachiopods (La Barbara, 1977) reorient actively when the current direction changes, certain sea anemones (Koehl, 1976), conical stalked hydroids, and erect bryozoans allow currents to orient them passively (Warner, 1977).

Monniot (1967) has reported that the subtidal ascidian *Microcosmos vulgaris* normally orients its siphons up-current, but is capable of actively reorienting when suspended material becomes too dense. Surprisingly, evidence for induced flow has been presented for only one ascidian species, *Styela plicata* (Bretz, 1972).

Styela montereyensis is a common stolidobranch ascidian in the Northeast Pacific. Johnson and Abbott (1972) have redescribed the species clearly, calling attention to morphological variation within and among local populations. The animal is anchored by an irregular tunic holdfast and held more or less erect in the water by a long stalk. Although both siphons are inserted anteriorly as in most ascidians, the larger incurrent siphon is recurved to point either posteriorly or ventrally. The entire body appears longitudinally plicated due to alternating thick and thin tunic areas.

Preliminary diving observations indicated that the flexible stalk of *Styela* allows ambient currents to reorient the animal passively, facilitating induced flow. The mechanism by which this occurs operates regardless of the direction from which the current comes. This feature is appropriate since *Styela* characteristically occupies shallow water where the surge oscillates, sometimes in unpredictable directions, every few seconds.

In this paper, we document current-induced flow and describe aspects of orientation, morphological variation, habitat utilization, and larval habitat selection related to the use of currents by *Styela*.

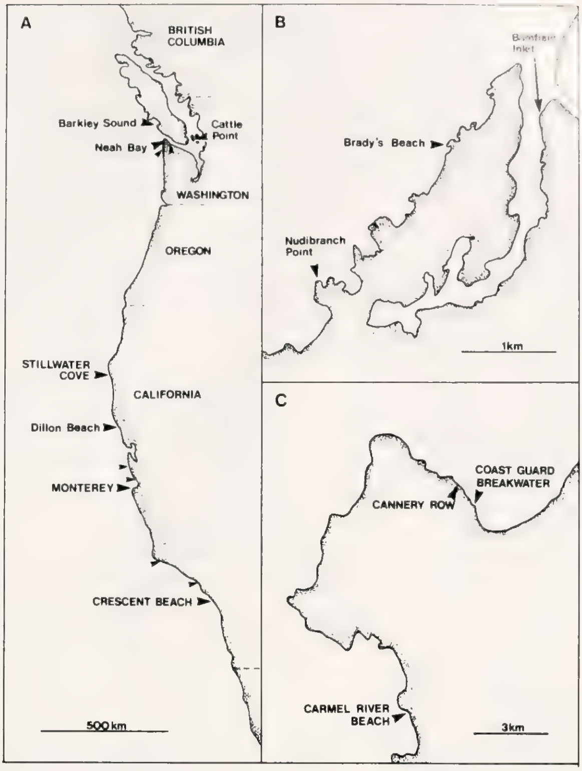

A
BRITISH COLUMBIA
Barkley Sound
Cattle Point
Neah Bay
WASHINGTON
OREGON
STILLWATER COVE
CALIFORNIA
Dillon Beach
MONTEREY
CRESCENT BEACH
500 km
B
Bamfield Inlet
Brady's Beach
Nudibranch Point
1km
C
COAST GUARD BREAKWATER
CANNERY ROW
CARMEL RIVER BEACH
3km

FIGURE 1. A) Known geographical range of *Styela montereyensis* on the Pacific Coast of North America, showing main study areas. Subtidal sites are capitalized. Unlabelled arrows indicate sites where *Styela* was not found. B) Mills Peninsula in Barkley Sound, British Columbia, showing 2 protected outer coast sites and a nearby protected site. C) Study sites on the Monterey Peninsula, California.

MATERIALS AND METHODS

We collected or observed *Styela montereyensis* at 11 sites from southern California to Vancouver Island (Fig. 1), thereby covering the species' known geographical range (Van Name, 1945). Although *Styela* characteristically occurs in areas of vigorous water movement (Fay and Vallee, 1979), its occasional appearance in quiet bays allowed us to compare morphology of specimens collected from a variety of current regimens. We made no quantitative measurements of current velocities, so our designations of sites as "open coast," "protected outer coast," or "protected bay" are based on subjective evaluation of local geography, surf, and fauna, following the criteria of Ricketts and Calvin (1962).

The nature of the substratum, approximate incline of the surface of attachment, orientation of the attachment surface relative to the direction of the prevailing surf or surge, depth or tide level, and approximate dimensions of the substratum were recorded for each individual we encountered while diving or collecting intertidally. Relative stalk length of each animal was determined in the laboratory by measuring the compression-resistant portion of the stalk and expressing this value as a fraction of the total body length exclusive of expanded siphons.

Living animals were transported in cold seawater (on ice, where necessary) to aquaria at Hopkins Marine Station, California; Friday Harbor Laboratories, Washington State; Bamfield Marine Station, British Columbia; or Brigham Young University, Utah. Larval cultures and live adults were maintained at 8°–14°C, depending on the season and the seawater system used.

At Neah Bay, Washington, we recorded the permanent orientations of ascidians relative to the horizontal. This was done at low tide by loosely holding each animal perpendicular to the piling, viewing it from the anterior end, and drawing an arrow on a slate to represent the direction the incurrent siphon pointed. The angles of the arrows were later measured using a protactor and placed in a "rose diagram" frequency distribution which was compared with a circular-normal distribution using chi-square (Batschelet, 1965).

Internal flow rates were estimated in a running seawater flume in which current velocity could be varied by regulating incoming water volume and the size of the outlet channel. Current speed in the tank was measured by repeatedly timing the passage of fluorescein dye along a 1 m course. Each animal was tied by its holdfast to a small weight in the flume, with the animal's incurrent siphon directed upstream. When the animal acclimated, about 1 ml of red food coloring (F.D.C. red #9,40) in seawater solution was introduced into the incurrent siphon with a pipette. A hand-held stop watch was used to measure the time elapsed before reappearance of the dye at the excurrent siphon. Twenty trials were made with each animal at each of several current speeds. The ascidians showed no adverse reaction to the dye, though they closed up in response to low concentrations of fluorescein or rhodamine.

To assess the possible influence of stalk flexibility on feeding, the rates of ingestion by animals free to sway with the currents were compared with those of animals restrained upright. Specimens of intermediate size (12 ± 2 cm) were collected from the Cannery Row site and held in clean seawater aquaria for 48 hr to clear food from their guts. Animals were paired by size (± 0.1 g, dry weight) and one of each pair randomly designated as the experimental animal. Each control animal was wired by its holdfast to the surface of a commercial structural brick, so that it could move freely. Each experimental animal was either anchored within a hole in the brick or caged above the brick in a narrow tube of 0.5 cm plastic mesh (Fig. 2). By restraining the experimental animals at two different vertical levels in this fashion, we hoped to control for boundary-layer and turbulence effects; some animals would feed at the vertical level of a flexed control animal while others would feed at the level of an erect control animal. The bricks with tunicates attached were placed in Monterey Bay at 4 m depth and left for 24 hr. They were then retrieved and brought into the laboratory, where the ascidians were removed and isolated in separate bowls of seawater. All feces expelled over the subsequent 24 hr were collected on pre-tared filter papers and washed with distilled water. Filter papers and animals were oven dried at 50°C and weighed on an analytical balance sensitive to 0.001 g.

Tadpole larvae were reared from spawned gametes or gametes removed by dissection. Responses of the settling larvae to light and gravity were assessed by examining the settling distributions of larvae in 16-ml-capacity molded-polystyrene petri dishes completely filled with water and covered on one side with black plastic tape (Young and Braithwaite, 1980). The dishes were cooled in a shallow seawater table under incandescent light at an intensity of 1500 lx.

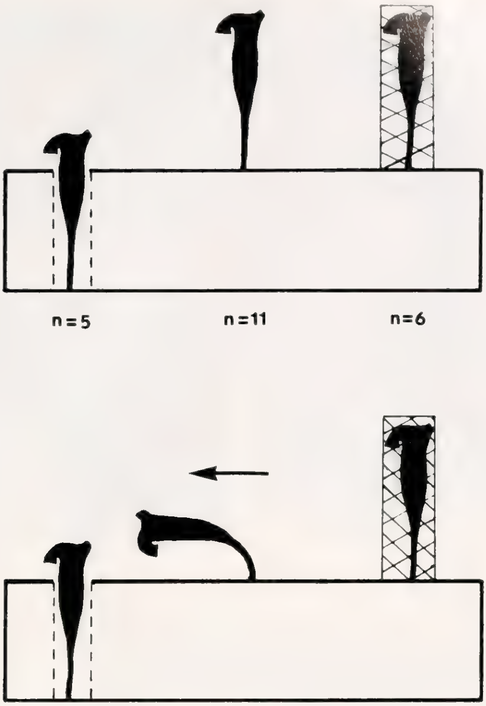
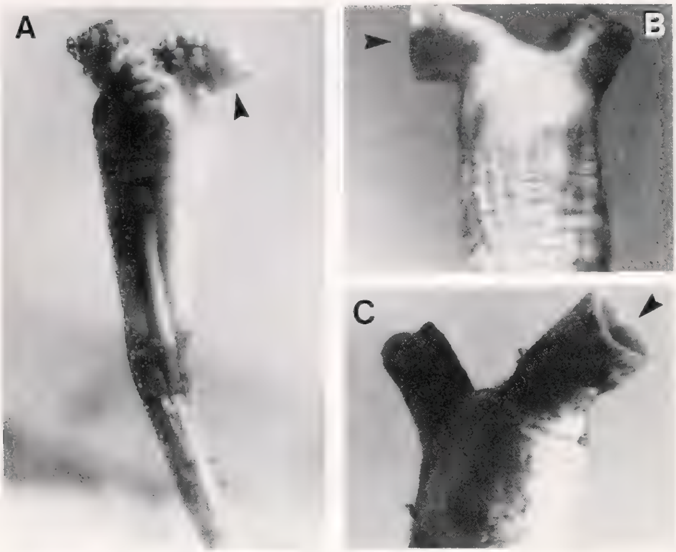


FIGURE 2. Schematic drawing of field feeding experiment, showing positioning of restrained and control animals in calm water (top) and current (bottom).

In a second experiment, larvae were placed in a circular channel, 5.75 cm wide and 3 cm deep, created by positioning a glass stacking dish in the center of a large shallow battery jar, half darkened with opaque black plastic. Twin air jets at opposite sides of the dish moved water and larvae around the channel at an average speed of 3 cm/sec. The tadpoles, slowly moving between the light and dark sides of the dish, were thus given the opportunity of settling anywhere along two continuous light–dark gradients, simulating conditions they might encounter in the field, where currents move them over illuminated and shaded portions of the substratum. After all animals settled, the distribution of zooids was plotted to the same scale on a large piece of paper. A protractor was used to divide the circular plot into 10° sectors. The number of animals in each sector was recorded, and each sector was paired with each opposite sector for a two-way analysis of variance in which the within-treatment variance tested the position of the sector pair relative to the air jets and the between-treatment variance tested light versus dark.

In a final series of experiments, groups of larvae were maintained in bowls of filtered seawater in continuous light or continuous dark. The dishes were monitored every few hours to assess the difference in settling times for larvae reared under these radically different lighting conditions. Yamaguchi (1970) and Crisp and Ghobashy (1971), working with other ascidian species, established precedent for this experimental design.



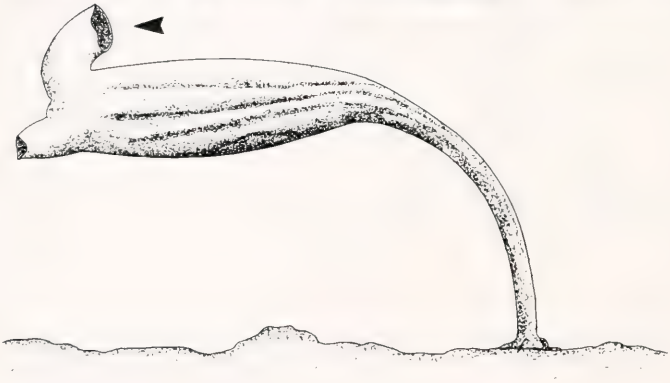


Figure 3. (Top.) Typical siphon inclinations of *Styela montereyensis* collected from the open coast (A) and protected bays (B, C). Arrows indicate incurrent siphon aperatures.

Figure 4. (Bottom.) Current-facilitated orientation in *Styela montereyensis*.

Results

Phenotypic plasticity

Two major morphological characteristics, incurrent siphon curvature and relative stalk length, varied among animals inhabiting different current regimes. By observing numerous animals in laboratory aquaria over periods of up to 2 months, we concluded that each consistently points its expanded incurrent siphon in a specific direction relative to its own longitudinal axis. The curvature which determines this direction apparently results from differential growth in the walls of the siphon. Specimens collected from all sites on the open coast or protected outer coast, either intertidally or subtidally, characteristically had siphons curved nearly 180° from the anterior point of insertion. The aperature was thus directed posteriorly. With few exceptions, animals collected from the two protected sites displayed ventrally-directed incurrent siphons (Fig. 3), as did some animals collected from deeper than 12 m in Monterey Bay.

TABLE I

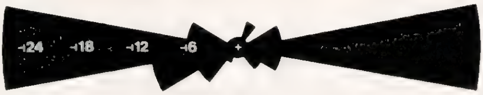
Relative stalk lengths of Styela montereyensis *at several sites (ranked subjectively by exposure). Variation among group means significant at* P < *0.001 (Cattle Point site not included in analysis because of small sample size). OC: open coast; PC: protected outer coast; PB: protected bay.*

Site	Exposure	N	Mean relative stalk length	Standard deviation
Stillwater Cove	OC	15	0.659	0.054
Barkley Sound	PC	14	0.655	0.066
Crescent Beach	PC	5	0.541	0.045
Breakwater	PC	50	0.572	0.066
Dillon Beach	PC	13	0.545	0.054
Bamfield Inlet	PB	9	0.470	0.031
Neah Bay	PB	80	0.388	0.061
Cattle Point	PB	2	0.461	0.033

Table I gives relative stalk lengths of animals from each site. Stalks are significantly longer in more exposed habitats, with the between-group variance component being significant at $P < 0.001$ by one-way analysis of variance.

Orientation and induced flow

Different orientation mechanisms are employed by *Styela* in habitats with different current regimes. In subtidal habitats with strong oscillating surge, *Styela* orients as follows: Each wave that passes onshore bends the flexible stalk far enough to align the longitudinal axis of the animal with the current. The subsequent backsurge instantly flips the animal 180° aligning it once again (Fig. 4). In either position, the recurved incurrent siphon points directly upcurrent. If the



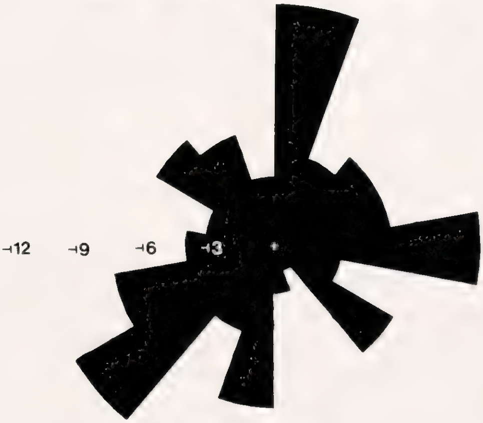


FIGURE 5. (Top.) Orientations of 95 *Styela* adults on vertical pilings in Neah Bay, as percentage of animals with incurrent siphon orientation falling within each 20° sector. Orientation is non-random ($P < 0.001$) by chi-square.

FIGURE 6. (Bottom.) Orientations of 95 juvenile *Stylea* (<3 cm long) on pilings at Neah Bay, shown as percentages as in Figure 5. Orientation is random ($P > 0.05$).

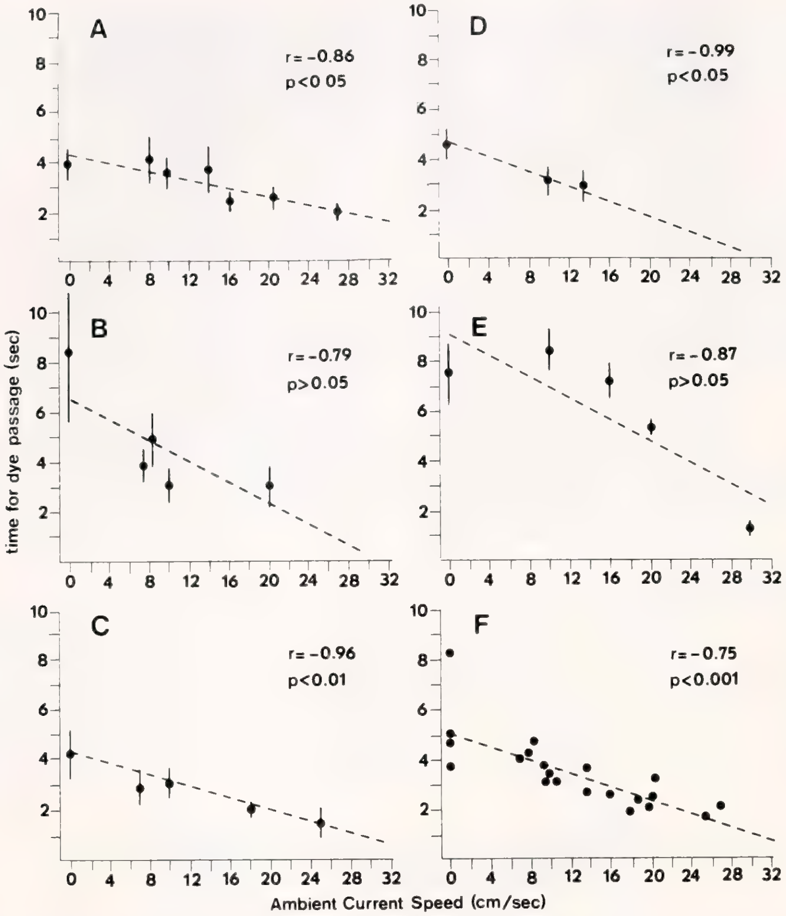


FIGURE 7. Relationship between current speed and internal filtering rate (measured by the time elapsed while dye passed from the incurrent to the excurrent siphon) in *Styela montereyensis*. Each point represents mean of 20 trials, shown with 95% confidence interval. A–D) 11 to 12 cm long animals. E) 15 cm long animal. F) Pooled data from A–D (shown without confidence intervals for clarity).

animal is anchored without nearby obstructions, orientation is effective regardless of the direction from which the current comes.

In quiet bays, the surge is seldom enough to sway the short-stalked animals. Individuals in Neah Bay attached to wooden pilings and floats display a permanent orientation. A significant majority of animals longer than 3 cm have their mid-saggital planes aligned parallel with the surface of the water (Fig. 5). Thus, the ventrally-directed incurrent siphons of most animals are pointed upcurrent (into either the surge or the backsurge) 50% of the time. Very small individuals are pointed randomly (Fig. 6), so the adult orientation apparently results from rotational growth rather than larval behavior or reorientation shortly after settling. Further indication of this is that the longitudinal furrows of Neah Bay specimens often spiral in the stalk region, while those of subtidal outer coast specimens most frequently run straight from the holdfast to the anterior end.

Table II

Fecal production by restrained and unrestrained Styela *in subtidal feeding experiment.*

Restrictive treatment	Animal dry weight (g):		Fecal weight (g)/Body weight (g):	
	Unrestrained	Restrained	Unrestrained	Restrained
hole	0.192	0.186	0.052	0.037
hole	0.408	0.286	0.046	0.017
hole	0.345	0.258	0.029	0.012
hole	0.332	0.256	0.057	0.004
hole	0.194	0.199	0.072	0.015
cage	0.175	0.167	0.068	0.036
cage	0.150	0.151	0.186	0.033
cage	0.214	0.229	0.168	0.030
cage	0.901	0.855	0.026	0.019
cage	0.767	0.741	0.027	0.013
cage	0.755	0.689	0.039	0.013

In laboratory flume experiments utilizing animals from Monterey Bay, flow through the branchial basket and atrium increased at higher current speeds. Furthermore, in most cases the relationship between external current speed and internal flow was significantly linear (Fig. 7). We augmented the number of points available for regression analysis by pooling data from all animals 11–12 cm long. Despite obvious behavioral differences between individuals, the points conformed to a linear model at the 0.001 level of significance. The maximum current speed tested with each animal was determined by the animal itself. Beyond a certain point, ranging from 14 to 30 cm/sec, each animal contracted its siphons and stopped filtering.

In ascidians, correlation between internal and external flow is not necessarily indicative of enhanced feeding, for two reasons. First, ascidians may be capable of increasing the activity of their lateral cilia when detecting current. Second, they are capable of filtering without actually feeding, by interrupting the secretion of endostyle mucus (MacGinitie, 1939). Our field experiment, in which ingestion (measured by fecal production) was compared in restrained and unrestrained animals, was designed to meet both of these potential objections.

All animals in the field experiments survived their respective treatments without apparent abrasion or other damage, and all animals fed while in the field. Within each pair of animals, the restrained animal always consumed less food (Table II). There was no consistant difference between the two restrictive treatments, so data from all pairs were pooled. Paired one-way analysis of variance revealed a significant difference ($P < 0.01$) between means of the restricted and control groups. Assuming all animals move food through their guts at a constant rate and that this rate is more or less independent of the volume of food ingested, we conclude that induced flow actually enhances feeding in the field. C. K. Goddard (University of New South Wales, personal communication, 1978), in using markers to trace the passage of food through the guts of other stolidobranch species, has shown both of the above assumptions to be true.

Habitat utilization

Styela montereyensis is found on hard substrata from the mid-intertidal to at least 15 m depth. Although the overwhelming majority of animals are attached to

TABLE III

Percentage of animals at each site free to sway horizontally in all directions, obstructed on one or more sides, or in surge channels.

Site	Intertidal (I) or subtidal (S)	No. man-dives or low tides	N	% Unobstructed within radius	% Obstructed within radius	% In surge channel
Breakwater	S	9	89	98.9	1.1	0.0
Cannery Row	S	4	29	100.0	0.0	0.0
Carmel River Beach	S	4	11	81.8	0.0	18.8
Stillwater Cove	S	3	21	66.7	0.0	33.5
Crescent Beach	S	3	9	0.0	100.0	0.0
Dillion Beach	I	3	14	0.0	21.4	78.6
Barkley Sound	I	3	13	23.1	23.1	53.8

large subtidal boulders or rock reefs, specimens of *Styela* are also found on a variety of other substrata including wooden pilings and floats, styrofoam floats, rubber tires, steel pipe, and cement. They occur as epizooites on the barnacles *Balanus nubilus* and *Balanus cariosus,* the mussel *Mytilus californianus,* the ascidians *Styela montereyensis* and *Pyura haustor,* and the algae *Cystoceira osmundacea* and *Gigartina californica.* In addition, some specimens at the Monterey Breakwater site are found anchored in sand. These latter animals have unusually large and ramified holdfasts for securing themselves in soft sediment.

Since surge currents are generally horizontal (Bascom, 1964), we hypothesized that animals on vertical surfaces might be in danger of battering against their substratum when the surge approaches head-on. Field observations support this hypothesis. Table III compares the number of animals at each site that are free to sway in any horizontal direction within their own radius with animals not free to sway. A significant majority of animals occupy the unobstructed positions, which are nearly always on the upward-facing surfaces of rocks or reefs. Animals on vertical surfaces are usually in surge channels where there are physical constaints on the direction of water movement.

At Crescent Beach, an extensive survey failed to reveal any specimens except on a single face, angled about 45° to the horizontal. Although the absence of an opposing face in close proximity precludes our classifying this site as a surge channel, we note that the attachment surface is aligned perpendicular to the beach in a small bay where the surf approaches from a single direction.

In the rocky intertidal, most animals occur either in surge channels or on the back sides of boulders, where water passes around the rocks on the backwash, creating effective surge channels where *Styela* orients by swaying in the usual fashion.

Larval settling behavior

We obtained abundant spawned or dissected gametes during every month of the year except March, July, and October, when we made no attempts. Embryogenesis (fertilization to hatching) takes 2 days (at 8°C) and the larvae may delay metamorphosis up to 6 days. Planktonic life may therefore be as long as 8 days.

Styela tadpoles are not strongly photonegative at settling. In eight replicate polystyrene dishes with an average of 100 tadpoles per dish, there was no significant preference for light or dark ($P = 0.844$; Wilcoxon signed-rank test). We ran two light-dark choice experiments in which larvae drifted passively around the dish with air-driven currents, one with 2441 tadpoles from Neah Bay and one with 183

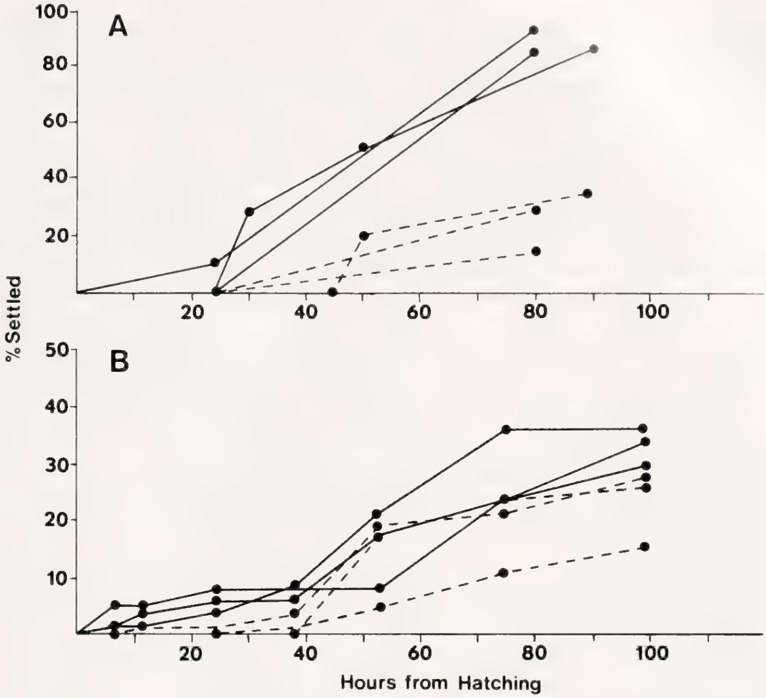


FIGURE 8. Settlement of *Styela montereyensis* larvae in complete darkness (dotted lines) and continuous light (solid lines). Each line represents a replicate run with 50 tadpoles. A) three experiments using tadpoles from Monterey Bay animals. B) three experiments using tadpoles from Neah Bay animals.

tadpoles from Monterey. The difference in settlement between light and dark was non-significant ($P < 0.5$, $P < 0.1$) by two-way analysis of variance, while the variance attributed to current effects was non-significant in the Neah Bay experiment ($P > 0.75$) and significant in the Monterey experiment ($P < 0.05$). This last difference is probably not due to a behavioral preference for a particular current regime; non-laminar flow in the dish apparently caused pooling in some regions, resulting in uneven settlement.

Styela larvae tend to be relatively lethargic, especially at the end of larval life when they generally rest on the bottom or drift passively rather than swim up in the water column. In the petri dish experiments, significantly more larvae settled on the bottoms of the dishes than on the undersides of the lids ($P = 0.008$; Wilcoxon signed-rank test). Ascidian species with more active tadpoles often show the opposite response in identical experiments (Young, unpublished data).

Both *Ciona intestinalis* (Yamaguchi, 1970) and *Diplosoma listerianum* (Crisp and Ghobashy, 1971) delay metamorphosis in continuous light. By contrast, *Styela montereyensis* tadpoles delay metamorphosis in continuous darkness (Fig. 8). This response is stronger in animals of the open coast than those of protected bays, but the reason for this difference is not known.

DISCUSSION

Among animals that utilize ambient currents for feeding, *Styela montereyensis* represents an extreme in adaptiveness. Its feeding mechanism is unusual in that efficient induced flow depends on precise orientation in habitats where current direction oscillates on a scale of seconds. *Styela* uses the force of the surge not only to induce flow, but also to effect the orientation that makes this current utilization possible. Thus, foraging may require little energy beyond initial growth and normal body maintenance.

Vogel (1978) has outlined three transducing mechanisms for inducing flow in biological systems: dynamic force, the Bernoulli effect, and viscous entrainment. At least two of these apparently play some role in the feeding of *Styela*. When *Styela* is aligned with the current, water is pushed through the feeding apparatus primarily by dynamic force against the incurrent siphon, but this effect is probably reinforced by viscous entrainment in which some water is drawn out of the excurrent siphon. In Neah Bay animals, dynamic force and viscous entrainment aid filtering when water comes from one direction only. On the backsurge, viscous entrainment should theoretically draw some water out of the incurrent siphon. We hypothesize that the effect of this entrainment is minimal and does not significantly offset the advantage obtained by part-time orientation.

Current utilization is enhanced in different habitats by the phenotypic plasticity of the species. Both open-water and protected bay forms are sometimes present in closely adjacent areas separated by a sharp exposure gradient. For example, the exposed beaches of Barkley Sound, British Columbia (Nudibranch Point and Brady's Beach), are only about 2 km from Bamfield Inlet and currents flow freely between the sites. Yet the animals at each site are distinctly different in form, There is no reason to suspect a barrier to larval dispersal, so differences are probably due to ecophenotypic (physiological) adaptation rather than genetic isolation.

Styela montereyensis rarely occurs in quiet water near the southern end of its range (Fay and Vallee, 1979). For example, in Newport Bay, California, where the stalked *Styela clava* has been introduced (Johnson and Abbott, 1972), *Styela montereyensis* is not found, despite the proximity of open water populations from which a bay population could be recruited. *Styela clava* has a ventrally- or anteriorly-directed incurrent siphon and a short stalk; it is almost identical in form to the Neah Bay *Styela montereyensis*. It is not known whether induced flow aids *Styela clava* in feeding or whether intrageneric competition excludes *Styela montereyensis* from Newport Bay.

Previous studies have shown that tadpoles of many ascidian species are photopositive at hatching or upon release from the parent colony, and then develop a strong photonegative response at the time of settling. This results in a preference for cryptic sites. By contrast, *Styela montereyensis* tadpoles in the dark delay metamorphosis for a short time. Beyond this, they are quite non-discriminating with regard to light or substratum. It is reasonable to suppose that photonegative behavior has been selected out of the behavioral repertoire of *Styela* as a maladaptive trait, since cryptic sites are probably the least suitable places for taking advantage of surge currents.

Despite the larvae's apparent non-discrimination, a large percentage of adults are found in unobstructed positions where they can orient freely. When animals die, their stalks often remain intact for a time. We have frequently found more stalks than animals on intertidal vertical surfaces, lending credence to the idea that the observed pattern of distribution results primarily from differential mortality following a nearly-random settlement.

Jorgensen (1955) has pointed out that most filter-feeders must expend a large proportion of their assimilated energy on food collection because of the relatively low concentration of usable particulate matter suspended in the sea. Because of its induced flow foraging method, *Styela* may be an exception to this generalization. At Neah Bay, animals recruited in the spring of 1979 grew to an average length of 13 cm by November. In Monterey Bay, we have found specimens as long as 32 cm, though Van Name (1945) reported the upper size limit of the species to be 20 cm. *Styela* has a lower basal metabolic rate than congeneric species found in quiet bays (C. Lambert, California State University, Fullerton, personal communication, 1978), and it is capable of spawning viable gametes in every season of the year. We suggest that a low energy demand for food gathering permits low metabolism and also frees energy for large size, indeterminate growth, and continuous reproduction.

Numerous stalked ascidians from the deep sea have recurved incurrent siphons (Van Name, 1945; Kott, 1969; Monniot and Monniot, 1978). Of special interest are species in several families with completely unciliated branchial baskets. The incurrent siphons of these forms are often hypertrophied. Although their modes of particle capture remain a mystery, Kott (1969) and Monniot and Monniot (1978) have independently speculated that bottom currents could greatly enhance their feeding by inducing flow. While *Styela montereyensis*, living in strong surge, has a strong branchial basket with small stigmata, the stigmata of deep sea forms are large, seeming to provide less resistance to the dynamic pressure of even slight currents. The fact that many of these species are found in shallower water only where there are strong upwelling currents (Monniot and Monniot, 1978) supports the hypothesis that they collect food in much the same way as *Styela*.

We wish to thank Dr. D. P. Abbott, D. Alexander, L. Cameron, Dr. C. K. Goddard, and G. Lambert for helpful discussions. Drs. J. R. Barnes, P. V. Fankboner, J. Farmer, R. L. Fernald, C. D. Jorgensen, and E. N. Kozloff critically reviewed various drafts of the manuscript. Laboratory space at Hopkins Marine Station and Friday Harbor Laboratories was provided by Drs. D. P. Abbott and A. O. D. Willows, respectively. We thank L. Cameron, R. Otto, A. Murray, and A. Rowe for serving as diving buddies and Dr. E. N. Kozloff for directing our attention to the Neah Bay population of *Styela*. This project was supported in part by a graduate internship award from Brigham Young University.

SUMMARY

1. Ambient water currents enhance internal flow and feeding in *Styela montereyensis* by forcing water through the branchial basket. Induced flow depends on upstream orientation of the incurrent siphon.

2. In open-coast and protected-outer-coast habitats, most animals occur in surge channels or on upward-facing horizontal surfaces where they sway freely with the surge. Orientation takes place as the flexible stalk bends with the passing of each wave, keeping the posteriorly-recurved incurrent siphon directed upcurrent.

3. In calmer water, animals' growth form is characterized by relatively shorter stalks and ventrally-inclined incurrent siphons. Adults in these habitats are mostly oriented with the mid-saggital plane parallel to the surface of the water. This orientation allows the ascidians to utilize the dynamic force in water surges coming from a single direction.

4. Observed patterns of microdistribution probably result from differential mortality rather than habitat selection. Larvae delay metamorphosis for a short time in complete darkness and show no strong preferences for shaded substrata. Otherwise, they seem almost non-discriminatory with regard to attachment sites.

LITERATURE CITED

BASCOM, W., 1964. *Waves and Beaches. The dynamics of the ocean surface.* Doubleday and Co., New York, 267 pp.

BATSCHELET, E., 1965. *Statistical methods for the analysis of problems in animal orientation and certain biological rhythms.* Amer. Inst. Biol. Sci. Monogr., 57 pp.

BRETZ, W. L., 1972. Effects of water current speed on the filtration rate of *Styela plicata,* a tunicate and epibenthic suspension feeder. *Am. Zool.,* **12**(4) : 720.

CRISP, D. J., AND A. F. A. A. GHOBASHY, 1971. Responses of the larvae of *Diplosoma listerianum* to light and gravity. Pp. 443–465 in D. J. Crisp, Ed., *Fourth European Marine Biology Symposium.* Cambridge Press, Cambridge, England.

CRISP, D. J., AND H. G. STUBBINGS, 1957. The orientation of barnacles to water currents. *J. Anim. Ecol.,* **26** : 179–196.

FAY, R. C., AND J. A. VALLEE, 1979. A survey of the littoral and sublittoral ascidians of Southern California, including the Channel Islands. *Bull. South. Calif. Acad. Sci.* **78**(2) : 122–135.

GRIGG, R. W., 1972. Orientation and growth form of sea fans. *Limnol. Oceanogr.,* **17** : 185–192.

JOHNSON, J. V., AND D. P. ABBOTT, 1972. The ascidians *Styela barnharti, S. plicata, S. clava,* and *S. montereyensis* in Californian waters. *Bull. South. Calif. Acad. Sci.,* **71**(2) : 95–105.

JORGENSEN, C. B., 1955. Quantitative aspects of filter feeding in invertebrates. *Biol. Rev. Camb. Philos. Soc.,* **30** : 391–454.

KOEHL, M. A. R., 1976. Mechanical design in sea anemones. Pp. 23–31 in G. O. Mackie, Ed., *Coelenterate Ecology and Behavior.* Plenum Press, New York.

KOTT, P., 1969. *Antarctic Ascidiacea.* American Geophysical Union Antarctic Research Series, **13** : 239 pp.

LA BARBARA, M., 1977. Brachiopod orientation to water movement I. Theory, laboratory behavior, and field orientation. *Paleobiology,* **3**(3) : 270–287.

MACGINITIE, G. E., 1939. The method of feeding of tunicates. *Biol. Bull.,* **77**(3) : 443–447.

MEYER, D. L., 1973. Feeding behavior and ecology of shallow-water unstalked crinoids (Echinodermata) in the Caribbean Sea. *Mar. Biol.,* **22**(2) : 105–129.

MONNIOT, C., 1967. Problemes ecologiques poses par l'observation des ascidies dans la zone infralittorale. *Helgol. Wiss. Meeresunters.,* **15** : 371–375.

MONNIOT, C., AND F. MONNIOT, 1978. Recent work on the deep-sea tunicates. *Oceanogr. Mar. Biol. Ann. Rev.,* **16** : 181–228.

RICKETTS, E. F., AND J. CALVIN, 1962. *Between Pacific Tides.* Third Edition, revised by J. W. Hedgpeth. Stanford Press, Stanford, Calif., 461 pp.

VAN NAME, W. G., 1945. The North and South American ascidians. *Bull. Am. Mus. Nat. Hist.,* **84** : 1–476.

VOGEL, S., 1974. Current induced flow through the sponge, *Halichondria. Biol. Bull.,* **147**(2) : 443–456.

VOGEL, S., 1978. Organisms that capture currents. *Sci. Am.* **239**(2) : 128–139.

WAINWRIGHT, S. A., AND J. R. DILLON, 1969. On the orientation of sea fans (genus Gorgonia). *Biol. Bull.,* **136**(1) : 130–139.

WARNER, G. F., 1977. On the shapes of passive suspension feeders. Pp. 567–576 in B. F. Keegan, P. O. Ceidigh, and J. J. S. Boaden, Eds., *Biology of Benthic Organisms.* Pergamon Press, New York.

YAMAGUCHI, M., 1970. Spawning periodicity and settling time in ascidians, *Ciona intestinalis* and *Styela plicata. Rec. Oceanogr. Works Jpn.,* **10**(2) : 147–155.

YOUNG, C. M., AND L. F. BRAITHWAITE, 1980. Larval behavior and post-settling morphology in the ascidian, *Chelyosoma productum* Stimpson. *J. Exp. Mar. Biol. Ecol.,* **42**(2) : 157–169.

ABSTRACTS OF PAPERS PRESENTED AT THE GENERAL SCIENTIFIC MEETINGS OF THE MARINE BIOLOGICAL LABORATORY

August 21–23, 1980

Abstracts are arranged alphabetically by first author within the following categories: Cell motility and cytoskeleton, comparative physiology, developmental genetics, ecology, fertilization and reproduction, microanatomy and microtechniques, molecular biology and biochemistry, neurobiology, parasitology and immunology, and plant pigments and photosynthesis. Author and subject references will be found in the regular volume index in the December issue.

CELL MOTILITY AND CYTOSKELETON

Labile components of the dogfish erythrocyte cytoskeleton. Diana C. Bartelt and William D. Cohen.

We used the dogfish (*Mustela canis*) erythrocyte to study the relationship between cell morphology and cytoskeletal structure and composition. Dogfish erythrocytes are flattened ellipitical cells with a nuclear bulge. Triton lysis under microtubule stabilizing conditions produces "cytoskeletons" consisting primarily of a nucleus, a continuous circumferential marginal band (MB) of microtubules, and a surrounding fibrous network (FN). The MB has been shown to be cold labile *in vivo* while the FN is cold stable. When lysates of cells at 0° and 25°C are examined by polyacrylamide gel electrotrophoresis (PAGE), proteins comigrating with a tubulin standard are augmented in the 0°C lysate but no material is found in either lysate comigrating with a spectrin standard. This is consistent with disappearance of the MB and stability of the FN. Dogfish erythrocytes undergo a Ca^{2+}-dependent change in morphology (from flattened ellipse to sphere) in Ringer's solutions containing ionophore A23187 and varying Ca^{2+} concentrations. Examination of the spherical cell "cytoskeleton" (phase contrast) indicates that both MB and FN are Ca^{2+}-labile *in vivo*. PAGE analysis of lysates from cells incubated in $A23187 + Ca^{2+}$ versus $A23187 + EGTA$ shows proteins in both tubulin and spectrin regions of the gel to be more prominent in the Ca^{2+} lysate, consistent with *in vivo* disassembly of both a tubulin-containing MB and a spectrin-containing FN. MB and FN of cytoskeletons prepared under our usual microtubule stabilizing conditions are stable at 0°C or in 5 mM Ca^{2+} (room temp.) over long periods. A major difference between the *in vivo* state and the *in vitro* conditions is the presence of KCl in the former. Inclusion of 150 mM KCl in lysing and washing media greatly enhances the *in vitro* lability of cytoskeletal components to both low temperature and Ca^{2+}. The MBs of cytoskeletons prepared and washed in the presence of KCl and 5 mM EGTA are labile to both 0°C and 5 mM Ca^{2+}. The FN is partially labile with long term exposure to 5 mM Ca^{2+} but stable at 0°C. Tubulin is present in both 0°C and Ca^{2+} extracts of these cytoskeletons, with spectrin appearing only in the long term Ca^{2+} extract.

Supported by NIH grant HL 20902 and CUNY grants 13313 and 13051.

Release of cytoskeletal proteins into the perfusate of squid giant axons. Jesse Baumgold and Paul Gallant.

Squid giant axons were used to study the effect of various physiological manipulations on the cytoskeletal proteins underlying the axolemma. The axons were intracellularly perfused and the bulk of the axoplasm removed using brief perfusion with a trypsin-containing solution followed by prolonged perfusion with a trypsin-free solution. The proteins dissolved in the perfusate were then analyzed by polyacrylamide gel electrophoresis after having been labeled with ^{125}I-Bolton-Hunter reagent. After prolonged perfusion, tubulin was the major protein eroded into the perfusate. The amount of protein released into the perfusate increased significantly following each of the following manipulations: potassium depolarization, electrical stimulation, and the application of 20–40 nM tetrodotoxin (TTX) to the external surface of the axon. The proteins released by potassium deplorization included tubulins, actin, 69K protein, and a number of minor ones. No neurofilament protein was detected. Application of TTX released the following proteins: tubulin, actin, 69K protein, 65K protein, a protein migrating between α and β tubulin, and several other minor ones.

Since the influx of calcium is dramatically increased when a nerve is depolarized or electrically stimulated, the mechanism underlying this release of cytoskeletal proteins during repetitive stimulation and potassium depolarization could involve this increased calcium influx. However, this mechanism cannot explain the release of protein in response to the application of TTX, since TTX does not increase the calcium influx. These experiments thus demonstrate that by manipulating the excitability of the giant axon, we can affect changes in cytoskeletal proteins. We therefore conclude that an intimate relationship must exist between the intrinsic proteins in the axolemma and the underlying cytoskeleton.

Light-scattering studies of sheared and unsheared actin polymerization. W. B. Busa, K. E. Van Holde, and M. S. Mooseker.

Light-scattering by polymerizing actin solutions was observed using a Perkin Elmer Model MPF-44 fluorescence spectrophotometer (excitation and emission wavelengths = 520 nm). Dogfish muscle actin (0.4 mg/ml) in 2 mM Tris (pH 8), 0.2 mM ATP, 0.5 mM dithiothreitol, and 0.2 mM $CaCl_2$ ws polymerized in the sample cuvette by addition of 50 mM KCl and 2 mM $MgSO_4$. Unsheared polymerization in the absence of ligands demonstrated a slow increase in the intensity of light scattered, reaching a plateau within 200 min. Polymerization in the presence of 18 μM phalloidin revealed an increase in rate of assembly but no change in the plateau. Cytochalasin B (5 μM) slightly increased assembly rate and significantly lowered the plateau.

The effect of shearing on assembly was observed by Pasteur pipetting five times at each of three 5-min intervals during polymerization. Shearing early during assembly caused a dramatic increase in assembly rate. Further shearing during assembly caused less, if any, increase. The plateau was achieved within 15 min, and equalled the plateau for controls.

Shearing *fully* polymerized F-actin caused a very rapid increase in scattering which decayed with time to the original plateau. This effect was not inhibited by phalloidin. Theoretical considerations indicate shearing *per se* should not alter scattering. The increase and decay observed may, however, reflect an effect of shearing and reannealing.

This work was performed under NIH Training Grant GM 00265.

The marginal band system of the dogfish erythrocyte. William D. Cohen, Deneene Whitehead, and Richard Jaeger.

The erythrocyte marginal band (MB) is a relatively simple and potentially useful microtubule system with which to approach problems in cellular morphogenesis and cytoskeletal function. We have been studying dogfish (*Mustelus canis*) erythrocyte MBs with respect to structure, function, isolation, composition, and biogenesis. Dogfish erythrocytes are relatively large, obtainable in quantity, and have the flattened, elliptical, nucleated morphology typical of non-mammalian vertebrate erythrocytes. When cells at room temperature (18°–22°C) are lysed with Triton X-100 under microtubule-stabilizing conditions, "cytoskeletons" consisting of MB, nucleus, and a sub-surface network enclosing the MB are obtained. Cells incubated at 0°C for 1 hr or more retain normal morphology. Lysis and thin sectioning show that MBs are absent but the network present, suggesting that the network maintains cell shape in the induced absence of the MB. MBs reassemble in living cells re-warmed about 1 hr. Colchicine does not induce MB disassembly, but prevents reassembly after 0°C-MB disassembly. MB influence on cell shape is evident in atypical erythrocytes with singly or doubly-pointed ends, which appear in cell suspensions stored at room temperature for long periods. Lysis reveals correspondingly pointed MBs, some with straight crossing extensions accounting for straight polar projections sometimes seen on living pointed cells. Points are lost and ellipticity regained in living cells at 0°C. This is consistent with the idea that cell morphology corresponds to that of a sub-surface network "set" for flattened, elliptical morphology, but subject to reversible deformation by the MB. Appearance of free (isolated) MBs in some cytoskeleton preparations has led to development of a mass MB isolation procedure. The isolated MBs are ribbon-like (TEM whole mounts) with inter-microtubule cross-bridges (thin sections). Recent observations on probable centriole participation in reassembly of an invertebrate (blood clam) erythrocyte MB prompted close examination of MBs in rewarming dogfish erythrocytes. Initial observations suggest that centrioles may function in vertebrate MB biogenesis as well, and raise the possibility that the plane of MB formation (plane of cell flattening) might correspond to the plane defined by a fixed right-angle centriole pair.

Supported by NIH grant HL 20902 and CUNY grants 13313 and 13051.

The theory of chemotaxis and the ability of a cell to sense its position and orientation within a tissue. R. P. FUTRELLE.

In embryogenesis, cells can move in coordinated ways while patterns of cytodifferentiation develop concurrently. Cells do this by producing and responding to chemical signals. It is possible to understand such chemical communication in fundamental terms (Bers, H. C. & E. M. Purcell, 1977, *Biophys. J.* **20**: 193–219). I'll give an example of chemotaxis, a critical part of cellular slime mold (CSM) development. Consider the orientation and chemotaxis occurring in response to N molecules emitted by a source cell and diffusing to a receiver cell. A concentration gradient develops at the receiver. The receiver counts the number of molecules hitting its two halves and finds a difference Δn, its orientation "signal." Due to thermal motion of the signal molecules, this count has an error, or "noise," given by the square-root-of-n law. The orientation will be reliable when the signal-to-noise ratio is > 1, or $N > (2r/d)^3$ with r the source-receiver separation and d the receiver cell diameter. This is a new kind of result which relates a *molecular quantity* N to the *macroscopic tissue geometry*. For example, if $d = 1/2$ mm, a typical embryonic field dimension, and $d = 10\ \mu m$, $N > 10^6$. Experiments show that CSM, leukocytes, and bacteria come close to the theoretical limit. $N = 10^6$ molecules corresponds to 4 μM in a 10 μm cell—not metabolically demanding. A cell can thus have many signal and receptor types and carry on many "pattern conversions" simultaneously.

Complex pattern formation is described by reaction-diffusion models. It will now be possible to estimate how many signal molecules a tissue needs to reliably generate a pattern. I have been able to reduce such models to the signal-cell paradigm by the mathematical technique of the functional derivative. This gives the perturbation in the pattern due to local cell function changes. Experimentally it corresponds to observing the effects of localized lesions or stimuli, an approach which has been successful in studies of CSM aggregation.

This research supported by NSF, under PCM79-04242.

Mitotic spindle behavior in unequal cleavage of Spisula solidissima. SHINYA INOUÉ AND KATŠUMA DAN.

In sea urchin eggs the first three cleavages are nearly equal. At the fourth division the animal cells cleave equally, but the vegetal four cells cleave unequally. Prior to division in the vegetal cells, the resting nuclei migrate to the vegetal pole where they attach themselves by a centrosome. Thus at nuclear envelope breakdown, the spindle is excentrically situated with two differently sized asters—a spherical proximal one and a distal flat or truncate one. In the zygote of *Spisula solidissima*, the two pronuclei migrate to the middle of the cell and immediately give rise to the definitive mitotic apparatus (MA); a resting nucleus is hardly found. In the zygote and CD cell of *Spisula*, the completed MA, first situated at the center of the cell, moves *in toto* to the egg periphery slightly higher than the equator. The distal end of the MA oscillates as though seeking the correct attachment site. The oscillation ceases and the spindle backs off from the cell cortex and proceeds through anaphase. Unequal cleavage ensues, as determined by the position and orientation of the early anaphase spindle. In the sea urchin egg, the nuclei themselves migrate to the vegetal pole; the nuclei and the centrosomes are already positioned, anticipating the micromere-forming division plane before the spindle is formed. In *Spisula*, the spindles are formed in the middle of the cell but are positioned and oriented by the onset of anaphase presaging the ensuing cleavage. The factor common to sea urchin and *Spisula* unequal cleavages seems to be neither the nucleus nor the spindle, but the centrosome. We shall turn to their behavior in relation to the cell surface or other determinants to look for the factors that initiate cell differentiation.

Grant support: NIH GM 23475-15, NSF PCM 81351.

Effects of cytochalasin B and phalloidin on F-actin and G-actin. MICHAEL P. MCCARTHY.

The effects of cytochalasin B (CB), and phalloidin on actin polymerization and F-actin steady-state viscosity were studied using Ostwald viscometry. Conventionally prepared scup and rabbit skeletal muscle actin was polymerized in 1 mM Tris-Cl, pH 8.0, 0.1 mM ATP, 0.25 mM DTT, 0.1 mM $CaCl_2$, 50 mM KCl, and 1 mM $MgSO_4$. As has been previously shown, actin polymerized in greater than stoichiometric amounts of phalloidin polymerizes more rapidly and reaches a higher steady-state viscosity than actin alone (Wieland, 1977, *Naturwissenschaften* **64**, 3037, whereas actin polymerized in substoichiometric amounts of CB reaches a lower steady-state viscosity (MacLean-Fletcher and Pollard, 1980, *Cell* **20**, 329). These changes can be correlated to altering the critical actin concentration below which actin won't associate; phalloidin lowers the critical concentration while CB raises it. The lower, CB-induced steady-state viscosity is the same regardless of whether CB is added before, during, or at steady-state polymerization. In competition experiments, the presence of phalloidin (25 μM), and CB (2 μM) during polymerization yields a steady-state viscosity intermediate between that of actin with phalloidin, and actin alone (7.5 μM). The intermediate steady-state viscosity induced by phalloidin and CB is also achieved by their addition to F-actin.

Supported by NIH Training Grant GM 00265.

In vivo *disassembly/reassembly of the marginal band in an invertebrate erythrocyte.* IRIS NEMHAUSER AND WILLIAM D. COHEN.

Erythrocytes of the Arcidae, like those of nonmammalian vertebrates and some invertebrates, are flattened and ellipitical and contain nuclei and marginal bands (MBs) of microtubules. The "blood-clam" erythrocyte MBs are notable for the association of each with a pair of centrioles. Furthermore, these MBs are cold labile (at 0–4°) *in vivo,* and reassemble with re-warming to 25°C. This system thus seemed ideal for investigating the possible role of centrioles in MB formation. Blood of *Noetia ponderosa* was diluted 1 : 10 in "Instant Ocean" artificial sea water and incubated at 0°C. After 1.5 hr, cell lysis with microtubule-stabilizing medium containing 0.4% Triton X-100 revealed the absence of MBs (phase contrast). Present were nuclei, centrioles, and remnants of other cellular organelles. Upon rewarming, the reassembled MBs once again were associated with the centrioles. Observation of the cells at intervals during rewarming reveals a developmental sequence: after 2 min centrioles are seen near the cell periphery, with fibers having free ends apparently emanating from them. After 5 min thin MBs have formed; they are pointed at one end with the centrioles located at the apex. After 15 min, the somewhat less pointed MBs appear to have thickened. By 120 min, MBs are observed with elliptical shape similar to that in controls. Thin sectioning (TEM) confirms the above results for untreated controls, and 0°C, 15-min-rewarmed, and 120-min-rewarmed cells. In addition, it shows that the centrioles are frequently in right-angle pairs and that the cell contents are enclosed by a fibrous network. After 15 min the reassembled MBs contain in cross section about 35 somewhat loosely packed microtubules (compared to about 50 tightly packed microtubules in controls). However, as in controls, the MBs are located in protrusions of the network, giving the impression that they could be producing tension in the latter. The 120 min rewarmed preparation is similar in appearance to the one obtained after 15 min, and reappearance of the MB in the former has been documented by electron microscopic examination of negatively-stained whole mounts. Experiments are underway to investigate further the 2 min time point, using immunofluorescence and electron microscopy.

Supported NIH grant HL 20902 and CUNY 13313 and 13051.

Vanadate inhibits ciliary beating in intact snail salivary glands. G. SALAMA, D. M. SENSEMAN, I. S. HORWITZ, AND B. M. SALZBERG.

In the salivary gland of the freshwater snail, *Helisoma trivolvis,* secretion may be regulated by the patterning of electrical activity generated by the effector neurons. To study the mechanism(s) coupling excitation to secretion, voltage dependent optical signals from different regions of the gland were detected with a 25 element photodiode array.

Intact glands were mounted on the stage of a microscope, stained with a solution containing 25–200 μg/ml Merocyanine-oxazolone dye (NK 2367), and then washed. Each element of the array recorded optical "spikes" at 680 ± 5 nm from 0.01 mm^2 of tissue. We observed higher than expected levels of optical noise, which we attributed to the oscillatory beating of the cilia that line the acini of the gland. In contrast to experiments on sea urchin sperm flagella, we found that sodium metavanadate, applied externally, blocked ciliary beating. In the presence of 10 mM vanadate, ciliary motion was reversibly blocked in 5 min. Occasionally, the presence of the vanadate enhanced the level of spontaneous electrical activity of the gland, and hyperpolarized the acinar cells by 5.0 ± 0.4 mV. The latter effects could be reversed in 5 min by returning the gland to vanadate-free Ringer's, in which the cilia remained immobile for more than 30 min.

Pharmacological effects resulting from the higher dye concentration could be prevented by a five-fold increase in Ca^{2+} concentration, to 20 mM, during staining. Consequently, staining the salivary glands in a 200 μg/ml solution of NK 2367 plus 20 mM Ca^{2+}, followed by vanadate treatment for 5 min, resulted in a 20-fold increase in the signal-to-noise ratio of the optical recording of membrane potential in this preparation. The large optical signals and the spatial resolution provided by the photodiode array can be used to study conduction pathways in this and other electrical syncitia.

The authors are grateful to Joel Rosenbaum and Dave Spray for suggesting the use of vanadate. This work was supported by NSF grant BNS 77-05025, NIDR grants De 05271 and DE 05536 and an M.B.L. Steps Towards Independence Fellowship to G. Salama.

The interaction of phalloidin with G- and F-actin. FRANCINE R. SMITH, K. E. VAN HOLDE, AND MARK S. MOOSEKER.

The interaction of phalloidin with actin from rabbit skeletal muscle was studied using sedimentation velocity and electron microscopy. Conventionally prepared G-actin contains a small population (3–5%) of oligomeric actin which may be removed by gel filtration, yielding "pure" actin (MacLean-Fletcher and Pollard, 1980, *Cell* **20**, 329). At low ionic strength (2 mM Tris-Cl, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$, pH 8.0 at 25°C), saturating amounts of phalloidin do not alter the sedimentation behavior of "pure" G-actin. Under identical buffer conditions, the addition of phalloidin to "conventional" actin slowly promotes the formation of two actin species ($S^{\circ}_{20,w} = 35S$, $S^{\circ}_{20,w} = 74S$) in addition to monomeric actin ($S_{20,w} = 3S$). The rapidly sedimenting species correspond to filaments of approximately 7 μ in length and to large aggregates, respectively. Similar species are observed in sedimentation velocity studies of actin polymerized by the addition of 50 mM KCl and 1mM $MgSO_4$ ($S_{20,w} = 31S$, $S_{20,w} = 41S$). Electron microscopic examination of filaments induced by phalloidin at low ionic strength indicates that they are morphologically identical to F-actin filaments polymerized by KCl and $MgSO_4$ addition.

Supported by NIH Training Grant GM 00265.

Observations on the isolated mitotic apparatus ghost. GEORGE W. SMITH.

A remnant of the isolated mitotic apparatus (MA) of eggs was partially characterized by electron microscopy and SDS gel electrophoresis. These mitotic apparatus ghosts (MAGS) were formed whenever the mitotic apparatus was depleted of its tubulin.

MAGS were formed by subjecting MAs (isolated by a modified microtubule polymerizing medium) to either low temperature (0°–4°C) or 1–100 μM of Ca^{2+}. They were isolated from four species of marine organisms and were morphologically indistinct from the regular MA when viewed under phase optics. Polarized light revealed a dramatic decrease in birefringence to about 15–20% of original in sea urchin and to almost negligible readings in the surf clam. Thin sectioning of MAGS revealed the absence of microtubules in the spindle or astral fibers. Microtubules were present in the centrioles. Oriented fibers, consisting of ribosomes and an amorphous material, were aligned where microtubules had been. It is thought that the residual birefringence of MAGS is due to this oriented material. SDS polyacrylamide gel electrophoresis revealed a normal MA pattern with about an 80% reduction in the tubulin band. High molecular weight proteins were also present but in amounts less than in a normal MA pattern.

No differences were noted between MAGS produced by cold or Ca^{2+} depolymerization with the exception that millimolar concentrations of Ca^{2+} frequently dissolved the entire MA.

Part of this study was supported by a Macy Foundation Grant to Marine Biological Laboratory.

Mitochondrial movements in early development of Ciona. DAVID STOPAK AND ROSARIA DESANTIS.

We have traced the movement of mitochondria during the development of the ascidian *Ciona intestinalis* by use of the laser dye Rhodamine 123. Previous studies have shown that mitochondria are concentrated after fertilization in the yellow crescent (myoplasm), which is segregated into the muscle lineage. Eggs were preincubated with 10 μg/ml Rhodamine 123, which specifically binds to mitochondria, and development was followed in the living embryo by fluorescence microscopy. In the mature egg mitochondria are already restricted to the vegetal 2/3 of the egg. The first shift from uniform distribution occurs at the time of germinal vescicle breakdown. The second dramatic shift occurs after fertilization, but only after meiosis is complete and the second polar body extruded. At this time the mitochondria form a posterior crescent just above the vegetal pole. By third cleavage the mitochondrial crescent is partitioned into the posterior vegetal pair of blastomers. The next two cleavages are unequal, occurring at the midpoint of the crescent. As early as the four-cell stage, however, a small clear protuberance forms at this location, suggesting that the cortex here may be different from other areas. By this method the muscle lineage can be followed into late gastrula. Other cells contain mitochondria at later stages, but the muscle lineage continues to emit the strongest fluorescent signal. To test whether cytoskeletal elements are responsible for crescent formation we have treated eggs with Cytochalasin B (2 μg/ml) and Colchicine (5×10^{-4} M), concentrations sufficient to block cleavage. We find that both drugs inhibit crescent formation. However, neither is completely effective. In many cases mitochondria appear concentrated near the vegetal pole but do not form a normal crescent.

We conclude that the movement of mitochondria is linked to both the microtubule and microfilament systems, but is not solely dependent on either one.

The mechanism of intra-plate ciliary synchrony in ctenophores. SIDNEY L. TAMM.

A ctenophore comb plate consists of hundreds of thousands of long cilia which beat together as a unit. In lobates (*i.e., Mnemiopsis*) the plates are triggered to beat by the interplate ciliated groove (ICG), which runs to the center of the base of each plate. A signal must therefore be transmitted outward from the ciliated groove junction to activate the beating of cilia on either side of the plate.

The nature of the synchronizing signal was investigated by microsurgical experiments on single comb plates of *Mnemiopsis*. A physical gap between two parts of a plate was created by holding back a sliver of the plate with a needle. Only the part of the plate connected to the ICG beat during passage of a wave; the other part did not beat. This effect was reversible: upon releasing the sliver, the entire plate beat as a unit. A mechanical block between two parts of a plate, without preventing the movement of an intermediate piece, was made by slitting a plate (but not the underlying tissue) with a razor blade. Again, only the side of the plate connected to the ICG was stimulated to beat, but both parts beat synchronously after the razor blade was removed. Finally, the tissue at the base of a plate was completely cut across, producing a narrow separation in the plate as well. Nevertheless, the two severed parts beat together. However, if the smaller part of the plate was moved away from the main part, the smaller part stopped beating. Upon allowing the two parts to come together, the entire plate resumed synchronous beating.

In ctenophores without an ICG (*i.e.*, cydippids, beroids, cestids), the beating of adjacent plates is triggered by hydrodynamic interaction between them, obviating the need for an intra-plate synchronizing mechanism. However, the first comb plate in each row is stimulated by the ciliated groove running from the aboral statocyst. Microsurgical experiments on the first plate in *Pleurobrachia* give results identical to those on lobates.

Thus, in cases where intra-plate coordination occurs, the cilia within a plate are synchronized by hydrodynamic coupling between them, not by cell-to-cell electrical transmission via the gap junctions between comb plate cells. The flange-like compartmenting lamellae of these cilia undoubtedly contribute to their mechanical coupling.

This research was supported by NIH grant GM 27903.

Binding of dynein to isolated meiotic spindles of the surf clam, Spisula solidissima. BRUCE R. TELZER AND LEAH T. HAIMO.

Recently, Haimo, Telzer, and Rosenbaum (1979, *Proc. Nat. Acad. Sci. U.S.A.* **76**: 5795–5763) demonstrated that flagellar dynein bound to and crossbridged microtubules assembled *in vitro,* and revealed intrinsic microtubule polarity. Experiments were undertaken to determine if dynein could also bind to native microtubules present within the mitotic apparatus. Dynein was isolated from axonemes of *Tetrahymena,* and meiotic spindles were obtained from activated eggs of *Spisula.* Isolated spindles were incubated in the presence of dynein for up to 60 min at 22°C and were collected by centrifugation. Analysis by gel electorphoresis demonstrated that dynein cosedimented with these spindles. The specific ATPase activity [μmol Pi/(min·mg)] of the dynein preparation alone equaled 0.20 while that of the spindles was 0.04. The activity of spindles incubated in dynein for 45 min was 0.24, indicating that dynein had bound. That the specific ATPase activity of the spindles containing dynein was higher than that of the soluble dynein indicated stimulation of the dynein's ATPase activity. In addition, spindles incubated in dynein exhibited greater stability and birefringence than those incubated in its absence. The retardation of spindles immediately after isolation was 1.44 nm. After 45 min spindles incubated in the absence of dynein exhibited little if any birefringence while those incubated with dynein exhibited a retardation of 1.00 nm, further supporting interaction of the dynein with the mitotic microtubules. Ultrastructral analysis is being undertaken to visualize the binding of dynein to the mitotic microtubules and, thus, to determine their polarity.

We are grateful to Joel L. Rosenbaum for generous use of laboratory equipment and for helpful discussions. We thank Ted Salmon and David Begg for suggestions and assistance. B.R.T. was supported by a Steps Toward Independence Fellowship. L.T.H. was a post-doctoral fellow of the American Cancer Society.

COMPARATIVE PHYSIOLOGY

Effects of temperature acclimation on nitrogen metabolism in two littorinid snails.

David W. Aldridge, Robert F. McMahon, and W. D. Russell-Hunter.

Temperature acclimation of nitrogenous excretion and oxygen uptake was investigated in a high littoral snail, *Littorina rudis* (=*saxatilis*), and the low intertidal species, *L. obtusata,* collected at Nobska Point, Massachusetts, and acclimated for 15–19 days at 4° or 21°C. Using an Orion ammonia probe, weight specific ammonia and urea (after urease treatment) excretion rates were determined based on tissue dry weight (TDW) as ng NH_3/(mg TDW·hr). Concurrent oxygen uptake rates (see Russell-Hunter, McMahon, and Aldridge, 1980, *Biol. Bull.,* **159**: 452) were determined with polarographic oxygen electrodes.

For *L. obtusata* (at 5 mg TDW) acclimated at 4°C total ammonia (NH_3+ urea as NH_3) excretion rates were 180 at 4°C; 159 at 11°C; and 363 at 21°C, yielding Q_{10} values of 0.84 (4°–11°C), 2.28 (11°–21°C), and 1.51 (4°–21°C). Rates for 21°C acclimated snails were 62, 182, and 312, yielding Q_{10} values of 4.66, 1.71, and 2.95, respectively. The mean urea NH_3 to ammonia NH_3 ratios for 4°C acclimated *L. obtusata* were 1.40, 1.29, and 1.01, respectively. These ratios for 21°C acclimated snails were 1.35, 0.73, and 0.46. Moles O_2 : moles NH_3 ratios for 4°C acclimated *L. obtusata* were 2.61, 5.97, and 4.52. These mole-ratios for 21°C acclimated snails were 6.41, 3.64, and 5.28.

For *L. rudis* (at 3 mg TDW) acclimated at 4°C, rates in ng NH_3/mg·hr were 151 at 4°C; 175 at 11°C; and 1325 at 21°C, yielding Q_{10} values of 1.23 (4°–11°C), 7.57 (11°–21°C), and 3.59 (4°–21°C). Rates for 21°C acclimated snails were 117, 179, and 897, yielding Q_{10} values of 1.84, 5.01, and 3.31. The mean urea NH_3 to ammonia NH_3 ratios for 4°C acclimated *L. rudis* were 1.82, 1.86, and 2.15, respectively. The ratios for 21°C acclimated snails were 1.39, 0.72, and 1.07. The O_2 : NH_3 ratios for 4°C acclimated *L. rudis* were 1.26, 3.74, and 1.51. These mole-ratios for 21°C acclimated snails were 2.19, 4.20, and 1.79.

There is no evidence for compensatory adjustments in excretion rates except for 4°C acclimated *L. obtusata* at low temperatures. The more aerial *L. rudis* excretes proportionally more urea than the more aquatic *L. obtusata.* Greater relative protein catabolism in *L. rudis* may reflect a dietary difference.

Supported by National Science Foundation research grant DEB-7810190 to Dr. W. D. Russell-Hunter, and funds from the Biology Department of The University of Texas at Arlington to Dr. Robert F. McMahon.

Janus green B: A vital stain during the process of mucus secretion.

B. G. Bang and S. J. Cooperstein.

The 80 μm urn cell complex of *Sipunculus nudus* responds to mucus-stimulating substances (MSS) *in vitro* by secreting streams of mucus from 4–6 loci of synthesis. Stock Janus-was a 1:10 seawater dilution of human serum heated at 85° for 4½ min. Added to urns, it induced tails of mucus 2–5× the length of the urn itself. Janus green B (JGB) is a blue dye comprising diethylsafranin linked to dimethylaniline by an azo bond. When added to urn cells the dye was bound by the synthetic loci of the cells and reduced, producing red-violet loci. When serum MSS was added, the emerging stream of mucus was blue. When secretion was stimulated before dye was added, the original secretion remained colorless but newly emergent mucus was blue. When JGB was reduced in a test tube and then added to urns, the loci did not bind the dye, and when MSS was added the emergent mucus was pink, indicating that the dye was bound to extracellular mucus. Janus green G differs from JGB only by having methyl rather than ethyl groups on the diethylsafranin moiety; it cannot be reduced. Added to urns, JGG stained the loci pure blue, and emergent MSS-stimulated mucus was colorless. Anaerobically, JGB killed most stimulated urns, but when glutathione was added to the MSS, urn cell death was prevented and long refractile blue mucous tails emerged. Together, results suggest that the synthetic apparatus of the urn cell complex binds the oxidized (not the reduced) form of JGB, which is then metabolically reduced; the reduced (not the oxidized) form is incorporated into the mucus granules during synthesis, and then is re-oxidized at exocytosis, probably by a component in the heated serum.

Supported by NIH 5 P50 HL 19157.

Thermostability of fish hemoglobins. THOMAS A. BORGESE, JOAN M. BORGESE, JOHN P. HARRINGTON, AND RONALD L. NAGEL.

The structural basis of protein heat stability is poorly understood, although electrostatic interactions (Perutz), hydrophobic interactions (Bigelow) or both (Kauzman) have been suggested. We have studied a set of homologus proteins (fish hemoglobins) and found that, as a group, their thermostability is lower than mammalian hemoglobins as measured by the time required for 50% precipitation of a 0.2 g/dl buffered hemoglobin solution at 50°C ($t_{\frac{1}{2}}$). By the same criterion, however, skate hemoglobin (type I) is more stable than human hemoglobin. When the pH dependency of thermostability was studied we found dogfish (*Mustelus canis*) hemoglobin (type II) to be stable at acid pH but unstable at alkaline pH. Conversely, toadfish hemoglobin (type III) is stable at alkaline but unstable at acid pH. Type IV hemoglobins (goosefish, tautog, ocean pout, scup, and sea robin) are unstable over the pH range (6.0–8.0) studied.

Thermostability of type I hemoglobin is unaffected by KCl concentration up to 1.0 M. Type II is stabilized and types III and IV destabilized by salt. All hemoglobins were progressively destabilized by alkylureas in direct relationship to the length of their hydrocarbon side chains (methyl < ethyl < propyl < butyl). All hemoglobins were stabilized when converted to the carbonmonoxy or cyanmethemoglobin form. The state of the heme group, in order of increasing effectiveness for thermostability is methemoglobin < oxyhemoglobin < carbonmonoxy and cyanmethemoglobin.

We conclude that (1) a few sequence changes in homologous proteins are sufficient to produce large differences in thermostability (2) the effect of salt, and consequently of electrostatic interactions, appears to be complex and variable for the four different hemoglobin types described (3) all fish hemoglobins were systematically destabilized when hydrophobic interactions were interfered with and (4) the ligand state of the heme and the strength of the heme to globin attachment are important determinants of hemoglobin thermostability.

Supported by PSC-BHE grants 12212 and 13321.

Mosaic development in the polyclad turbellarian Hoploplana inquilina *and its evolutionary implications.* BARBARA BOYER.

Spiral cleavage and mosaic development occur together in annelids and molluscs. My earlier work, however, showed that the acoel turbellarian embryo, with duet spiral cleavage, is regulative, suggesting that spiral cleavage and mosaicism originated independently in evolutionary history. In this study cell deletions were done on the polyclad *Hoploplana inquilina* to determine if the embryos of this closely related turbellarian order with typical quartet spiral cleavage are regulative or mosaic.

Single blastomeres were deleted at the two-cell stage by puncture through the egg membranes with tungsten needles. Of 70 complete deletions, 33 survived to form larvae which were compared with the Mullers-larva controls for such characteristics as general morphology, lobe development, number and position of eyes, and swimming behavior. In all cases the experimental larvae were abnormal, exhibiting a shape suggestive of the Mullers larva but with rudimentary lobes, only one or no eyes, and aberrant swimming behavior. Most significantly 33% of the experimental larvae were eyeless and 51% had only one eye suggesting a difference in the morphologenetic capacities of the blastomeres at the two-cell stage and possible interaction between these blastomeres in normal development to form two eyes.

The results demonstrate that the polyclad *Hoploplana* is mosaic with half embryos always forming deficient larvae and suggests that mosaicism became associated with spiral cleavage in the quartet form during the evolutionary history of the Turbellaria. This association has evidently remained a permanent feature of the turbellarian-annelid-mollusc lineages.

This work was supported by an MBL Steps Toward Independence Fellowship and NSF grant PCM 77 16269 to Dr. John Arnold.

Substitution of calcium by polycations in sponge aggregation factor interaction. WERNER BURKART AND MAX M. BERGER.

Aggregation factor (AF) from the marine sponge *Microciona prolifera* promotes species-specific reaggregation of *Microciona* cells. Although highly specific binding of AF to cells is calcium independent, AF mediated cell aggregation probably based on AF–AF interactions needs high calcium concentrations of 10–20 mM.

Using AF-coated Sephadex Superfine beads and iodine-125 labeled AF to assay AF–AF interaction, it was shown that minute amounts of polycations like polylysine, histones, or P-120 can substitute for calcium. As a control, low background binding of AF to bovine serum albumin derivatized beads was shown not to be affected by these polycations. The concentrations needed to get binding as high as with seawater concentration of calcium (450 μg/ml = 10 mM) were about 1 μg/ml for P-120 and histones and 10 μg/ml for polylysine. Based on a comparison of charges, the polycations exert their effect at a charge concentration which is 10,000× lower than that needed for calcium. Spermidine, with only three charges per molecule at concentrations of up to 30 μg/ml, had no effect on AF–AF interaction. Neither did polyanions like hyaluronic acid or chondroitin sulfate interfere with the binding of iodine-125-labeled AF to AF-carriers in the concentration range tested.

In order to measure the interaction of single AF molecules with polycations and calcium, the distribution of iodine-125-labeled AF in a two-polymer aqueous phase system composed of dextran T500 and polyethylene glycol 6000 was measured. By charging the system, the huge AF molecule ($20 \cdot 10^6$ dalton), having polyanion characteristics at neutral pH, is driven into the upper phase. Addition of 1 μg/ml polylysine or histones inverts the effect, suggesting the formation of complexes having a net positive charge. The most potent polycation, P-120, having more widely spaced charges, brings the partition coefficient near 1 over a wide concentration range by just neutralizing the AF-molecule.

Based on these results, we propose that large areas with a multitude of charged binding sites are involved in AF–AF interaction. Although the specificity of the single binding site would be very low, cooperative effects could still provide enough selectivity to ensure species-specific AF–AF interaction and, together with the high binding specificity of AF to the cell surface, cell sorting.

This work was supported by Swiss National Science Foundation grant 3.513-079.

Gas secretion in the swimbladder of shallow water fishes compared to a deep ocean fish (3000 meters). EUGENE COPELAND, RICHARD HAEDRICH, AND JONATHAN WITTENBERG.

By scanning electron microscopy, the gas-secreting epithelia of the swimbladder of five shallow water fish (cunner, toadfish, *Fundulus*, spadefish, and bluefish) are compared to that in the deep sea (3000 m) rat-tail fish, *Chalinura* (*Coryphaenoides*). The shallow water fish show evidence of a bubble release from a relatively smooth surfaced epithelium, as postulated earlier at the transmission electron microscope level (Copeland, 1969, *Zeit. Zellforsch.* **93**, 305–331). The appearance of the surface of the secretory epithelium in *Chalinura* is completely different. The capillaries of the rete mirable enlarge and loop onto the surface as expanded tubules whose surfaces are bound by circular ridges (like galvanized drain pipe). There is no sign of bubble release under conditions when free gas is secreted against some 300 atm pressure. Conclusion: either the interpretation of the mechanism of gas release in the shallow environment is at fault or the very deep-living fish have a different mechanism.

L-Glutamate–specialist chemoreceptors on the legs of the lobster Homarus americanus. CHARLES DERBY AND JELLE ATEMA.

Lobsters taste with chemoreceptors on their legs. These chemoreceptors function in food location and consumption. Multi-unit neurophysiological recordings from leg chemoreceptors demonstrated that, among the 48 compounds tested, L-glutamate, hydroxy-L-proline, and ammonium chloride, in that order, are the most stimulatory. L-Glutamate-sensitive cells were studied in more detail by using single-unit extracellular techniques, in order to determine their threshold and specificity.

L-Glutamate-sensitive cells have thresholds for L-glutamate near 5×10^{-8} M. This cell type responds with less than 8% of its L-glutamate response to 19 other equimolar compounds. Any change in the L-glutamate molecule causes a drastic reduction in the degree of excitation of these cells: Alteration of the carbon chain length (DL-α-aminoadipic acid, L-asparatate), alteration of either of the carboxyl groups (L-glutamine, γ-amino-n-butyric acid, L-glutamate-γ-methyl ester, norvaline, 4-amino-n-valeric acid), substitution on the α-carbon (DL-α-methyl-glutamate), addition of a second or third amino acid (L-glutamyl-L-glutamate, glutathione), isomerization (D-glutamate), deamination (glutarate), and cyclization (L-pyroglutamate). These cells also do not respond to other compounds which, in multi-unit recordings, are very stimulatory; these compounds include hydroxy-L-proline, ammonium chloride, L-arginine glycine, betaine, and taurine.

Thus, there are L-glutamate-specialist chemoreceptors in the taste organs of lobsters. Since L-glutamate is one of the common free amino acids in the tissues of many prey species of lobsters, these L-glutamate specialists are probably involved in detection and identification of food items.

Thrust and drag of bluefish (Pomatomus saltatrix) *at different buoyancies, speeds, and swimming angles.* ARTHUR B. DUBOIS AND CHRISTOPHER S. OGILVY.

Factors which affect the thrust and drag of swimming fish were studied using a water circuit in which bluefish averaging 52 cm and 1.53 kg were placed. Thrust and drag (F_D) in Newtons were calculated from Newton's law using the mass of each fish and the acceleration and deceleration recorded from a miniature accelerometer implanted near the center of gravity. Speeds were varied between 0 and 1 m per sec. Buoyancy was adjusted to neutral (about 75 cc), then between 45 cc negative and 15 cc positive buoyancy, by changing the volume of air in a balloon implanted in the swimbladder. The angle of inclination of the tunnel with respect to horizontal was varied between 23° head down and 15° head up. The regression line for mean drag on speed during neutral buoyancy, which was independent of angle, in nine bluefish was: $F_D = (0.51 \times \text{speed}) + 0.15$, $r = 0.73$, S.E. of estimate (E) 0.090, in kg m/S^2, over the range 0.15–0.95 m/S. At 30 cc negative buoyancy, horizontal, $F_D = (0.26 \times \text{speed}) + 0.31$, S.E. of E 0.008, over the range 0.31–0.70 m/S, where 0.31 is stalling speed. With buoyancy negative 30 cc, head down 17°, $F_D = (0.72 \times \text{speed}) - 0.011$, S.E. of E 0.023, over a range 0.31–0.90 m/S. Negative buoyancy 30 cc, head up 15°, $F_D = (0.31 \times \text{speed}) + 0.29$, S.E. of E 0.005, at a range 0.31–1.3 m/S. With 15 cc positive buoyancy, 9° head down, $F_D = (0.68 \times \text{speed}) + 0.039$, S.E. of E 0.072 in the range 0.23–0.82 m/S. The bluefish were able to use their pectoral fins, whose mean projected area was 34.5 cm^2, to sustain their weight in water at speeds above stalling speed, but at the cost of extra energy. They would not actually glide downward, since the force of drag, F_D, exceeded the force of their weight in water multiplied by the sine of the glide angle.

Relationship of body colors to environmental light conditions in "poster colored" fishes. JOSEPH S. LEVINE AND EDWARD F. MACNICHOL, JR.

Marine and freshwater fishes exhibit great variety in their phototopic visual pigment systems. Some of the variations in wavelengths of peak absorption may be related to water color in the habitat. Additional differences are related to the unique visual tasks facing each species, including visually mediated intra-specific communication, where color cues are often important. In an analysis aimed at describing visual communication quantitatively, working hypotheses were: 1) Contrast between adjacent black and white areas should be highly conspicuous under most conditions; 2) The range of hues useful in generating color contrast should narrow with depth as the spectral-band width of the available illumination decreases; 3) The specific colors useful as conspicuous markers should be predictably different in water with different transmission characteristics.

Colors in high-quality photographs of fishes were quantified using chips from the Munsell Book of Color. All colors—and the achromatics white, black, grey, and silver—were sampled along six body transects. Total and relative frequencies of occurrence and areas covered were combined to obtain an importance value for each color. One study group included all Hawaiian members of five coral reef families, divided into shallow (≤ 20 m) and deep water (> 20 m) species. The other was South American (reddish-brown water) and African (blue water) cichlids. In both groups, black and white were the most important colors. Among actual hues, blue and yellow were most common among blue water species, while green and red predominated in the red-brown water forms. Contrary to predictions, deep water reef fishes showed minimal decrease in the importance of red coloration compared to shallow-water confamilials. However, red and orange are visually equivalent to black in the blue deep-water environment, and may be relatively easy to concentrate from ingested algae and invertebrates, whereas melanin must be synthesized from tryosine, possible with a greater energy requirement.

Respiration in the polychaete worm Magelona: *reponses to temperature, hypoxia and tentacle ablation.* ROBERT F. McMAHON AND W. D. RUSSELL-HUNTER.

Magelona sp., a small polychaete worm (family Magelonidae) with the unusual blood pigment, haemerythrin, burrows in sands of low oxygen content in the shallow sublittoral. The pair of feeding tentacles, extended from the burrow, apparently function in gas exchange in the well-oxygenated waters just above the substratum. Living specimens of *Magelona* were collected from Stony Beach, Woods Hole, Massachusetts, during the summer of 1980. Polarographic oxygen electrodes were utilized to record oxygen consumption rate continuously as $\dot{V}O_2$ [μl O_2/(mg dry tissue weight·hour)]. Uptake rates were recorded with decreasing oxygen concentrations at 20°C from near air saturation for oxygen ($PO_2 = 159$ torr) until uptake ceased at 1–10 torr, and at near air saturation from 10°–45°C in 5°C intervals both for whole individuals, and for those with tentacles removed.

Magelona appears adapted to reducing sands. It is a good regulator of $\dot{V}O_2$ with a critical PO_2 of 56–60 torr. Tentacle ablation has no significant effect ($P > 0.1$) on $\dot{V}O_2$ below 140 torr although intact specimens maintain slightly elevated $\dot{V}O_2$'s over 140–160 torr. Mean $\dot{V}O_2$ was 0.460 μl O_2/(mg·hr) at 10°C and increased to 2.026 μl O_2/(mg·hr) at 30°C. Q_{10} values for each 5°C increase from 10° to 30°C ranged from 1.88 to 2.43. $\dot{V}O_2$ and Q_{10} values in specimens without tentacles were very similar to those of intact specimens over 10°–30°C. $\dot{V}O_2$ increase markedly to 7.648 μl O_2/(mg·hr) at 40°C, with Q_{10} values for 30°–35°C being 5.04 and for 35–40°C being 2.83; and declines at 45°C, which is lethal to *Magelona*. The $\dot{V}O_2$ of specimens without tentacles was significantly ($P \leq 0.1$) inhibited at higher temperatures, being only 56–70% that of intact specimens at 35° and 40°C, respectively (Q_{10}'s: 30°–35°C; 2.57 and 35°–40°C; 3.57). This reduction of $\dot{V}O_2$ in specimens without tentacles occurs under the elevated oxygen demand induced by temperature stress. Therefore the tentacles of *Magelona*, which are only 2–3% of the dry weight, appear to function as respiratory surfaces.

Supported by National Science Foundation research grant DEB-7810190 to Dr. W. D. Russell-Hunter, and funds from the Biology Department of The University of Texas at Arlington to Dr. Robert F. McMahon.

Interstitial fluid pressures of smooth dogfish (Mustelus canis) *and bluefish* (Pomatomus saltatrix) *titled in air.* CHRISTOPHER S. OGILVY AND ARTHUR B. DUBOIS.

Interstitial fluid pressure (IFP) can be measured using a cotton wick and polyethylene tubing (PE 160) inserted subcutaneously. One was inserted in the head region and another in the caudal region of live, unanesthetized dogfish and bluefish. The fish were placed horizontally on a V-board in air while the gills were perfused with sea water. The five dogfish tested had an average pre-tilt head IFP of 2.0 cm H_2O (S.E. ± 2.3) and a caudal IFP of 2.9 cm H_2O (S.E. ± 1.9). The fish were tilted head up to 30° and IFP in the caudal region rose to 15.8 cm H_2O (S.E. ± 7.4). The head IFP only rose 0.3 cm H_2O. After 30 min of tilt the fish were lowered. At the end of a 30 min follow-up period the head IFP was 2.5 cm H_2O (S.E. ± 1.8) while the caudal IFP was 2.6 cm H_2O (S.E. ± 1.2). In four bluefish the average pre-tilt head IFP was 0.4 (S.E. ± 0.2). During the tilt the head IFP rose to 2.1 cm

H_2O (S.E. ± 0.9) while the caudal IFP rose slightly to 0.8 cm H_2O (S.E. ± 1.2). After the tilt the IFP in the head was −0.6 cm H_2O (S.E. ± 1.5) and the caudal IFP was −0.1 cm H_2O (S.E. ± 0.9). These results show that when a dogfish is tilted head up in air, fluid enters the interstitial space in the caudal region. This is reflected by a large positive IFP. Because only a slight increase in caudal IFP is observed in bluefish, we conclude they can compensate for the tilt. The dogfish tested typically showed blood oozing from the tail and died a few hours after the tilt, whereas the bluefish survived well. Perhaps the hydrodynamic forces acting on the fast swimming bluefish pre-adapted this animal to tolerate gravity while the slower swimming dogfish has much less adaptation.

Lack of respiratory response to temperature acclimation in two littorinid snails.

W. D. RUSSELL-HUNTER, ROBERT F. MCMAHON, AND DAVID W. ALDRIDGE.

Rate functions of physiological processes, such as oxygen uptake, may adapt to temperature changes over three distinct time-scales: (a) directly, within minutes or hours, (b) by compensatory acclimation over days or weeks, and (c) by natural selection over many generations. The commonest snail of the midlittoral, *Littorina littorea,* shows some thermoregulation of respiration (McMahon and Russell-Hunter, 1977, *Biol. Bull.,* **152**: 182–198; Newell and Roy, 1973, *Physiol. Zool.,* **46**: 253–275) but little acclimation. The high littoral snail, *L. rudis* (=*saxatilis*), and the low intertidal species, *L. obtusata,* differ from *L. littorea* in their temperature responses. Thus possible acclimation was investigated in conjunction with simultaneous studies on excretion. Both species were collected at Nobska Point, Massachusetts, and acclimated (at 4° or 21°C) for 15–19 days.

For each test temperature (4°, 11°, 21°), oxygen uptake rates were based on 10–15 determinations using polarographic oxygen electrodes, and computed from log–log regressions against tissue dry weight (TDW) to give $\dot{V}O_2$ in $\mu l\ O_2/(mg \cdot hr)$. Such $\dot{V}O_2$ values for *L. obtusata* (at 5 mg TDW) acclimated to 4°C were: 0.620 at 4°C, 1.256 at 11°C, and 2.157 at 21°C, yielding Q_{10} values of 2.74 (4°–11°C), 1.72 (11°–21°C) and 2.08 (4°–21°C). For 21°C acclimated *L. obtusata,* corresponding $\dot{V}O_2$ values were: 0.530, 0.872, and 2.163, yielding Q_{10}'s of 2.04, 2.48, and 2.29. Corresponding $\dot{V}O_2$ values for *L. rudis* (at 3 mg TDW) acclimated to 4°C were 0.250, 0.863, and 2.626, yielding Q_{10}'s of 5.87, 3.04, and 3.98. For 21°C acclimated *L. rudis,* corresponding $\dot{V}O_2$ values were 0.339, 0.988, and 2.116, yielding Q_{10}'s of 4.61, 2.14, and 2.94.

Irrespective of acclimation history, *L. rudis* shows unusually high Q_{10} values over the 4°–11°C range. Neither species shows any significant difference in $\dot{V}O_2$ ($P > 0.05$) between 4° and 21°C acclimated individuals at any test temperature. It seems possible that this lack of an acclimatory response is an adaptation to the wide short-term temperature variations experienced on rocky seashores. These two littorinid species are exposed to more marked diurnal and semilunar environmental changes than are nonmarine and deeper marine forms whose capacity for acclimatory compensation has evolved to fit sustained longer-term temperature changes.

Supported by National Science Foundation research grant DEB-7810190 to Dr. W. D. Russell-Hunter, and funds from the Biology Department of The University of Texas at Arlington to Dr. Robert F. McMahon.

Stability of dogfish lens fiber cell membranes.

SEYMOUR ZIGMAN, TERESA PAXHIA, AND TERESA YULO.

Lens fiber cells may be the largest and most stable cells present in soft vertebrate tissues. While the hardness and elasticity of the lenses of different vertebrates varies remarkably, the basic units comprising most of the substance of the lens are quite similar. In the nucleus of the lens, these are organized (by many strong gap junctional connections) into closely contiguous membranes that contain excesses of extrinsic lens proteins. Nearly all cytoplasm and cell particles are absent from these highly differentiated cells.

After sucrose-gradient (5–20%) purified fibers were extracted with tris-buffer (pH 7.2) 8 M urea, and 1% SDS, scanning EM revealed that the basic ribbonlike appearance of these fibers in *Mustelus canis* remained intact. Changes observed were fiber thinning and the removal of the characteristic interdigitating knobs.

While the peptides dervied from the proteins extracted from the lens membranes were common to the total soluble proteins, as shown by SDS-PAGE, two bands were present only in the membrane extract (55,000 and 45,000 daltons). These were lost when DTT was used in the extraction solvent, indicating that -SS- bonds are involved in crosslinking. Membrane lipid analyses showed the following: protein : lipid of 5 : 1; PL : C + CE of 2.5, fluidity 28%; PE = Sph > other PL. The combination of the physical stability of the lens fiber cell membranes and the continued binding of formerly soluble lens proteins to them may explain the conservative process of lens nucleus growth and hardening during aging.

Grant support: NIH (EY00459); Pledger Fund; Mullie Fund; RPB, Inc.

DEVELOPMENTAL GENETICS

Healing of Xenopus *eye fragments premarked by chimaeric pigment grafts.* Kevin Conway and R. Kevin Hunt.

Albino (alb^{P}) Xenopus laevis embryonic (stage 24–28) eyebuds were marked with small orthotopic grafts from normally pigmented donors. About a day later (stage 33–37) half the eyebud was deleted, leaving a locally marked half-bud fragment. These fragments were observed and photographed as they rounded up to form whole small eyes in the post-operative days, and then normally sized eyes in the following weeks of tadpole life. Marked dorsal, ventral, anterior, and posterior fragments were prepared, each type (*e.g.* dorsal) bearing one of three types of marker grafts (*e.g.* posterior, dorsal, or anterior). In the first post-operative day, marked tissue near the cut edge migrated along the cut edge, except for ventral tissue, which did not move, presumably due to the ventral fissure. Marked tissue away from the wound did not move much in the first day. However, in the succeeding days, anterior marks in anterior fragments moved dorsally, and increased their angular extent. Similarly, posterior marks in posterior fragments moved dorsally, and expanded. Dorsal marks in dorsal fragments and ventral marks in ventral fragments expanded without moving. The clonal configuration did not change substantially a week after the operation, and was elaborated radially as the eye grew, in a manner similar to the normal growth of eyebuds with orthotopic pigment-marked clones. Thus the healing of eyebud fragments is asymmetric, in that ventral tissue is not free to contribute to the early (Day 1) melting of tissue into the wound. Also, the healing is not strictly an epimorphic regulation accomplished only by cells at the wound edge. As cells far from the cut move and expand, some morpholactic mechanism must instruct them to change their fate.

This work was supported by an NIH Training grant (GM 07231) to K. Conway, and NSF (PCM-77-26987) to R. K. Hunt.

Pigmentation mosaicism in the choroid of the eye after embryonic grafting. R. Kevin Hunt, Steve Eckman, and Kevin Conway.

An interesting assymetry exists when half eye-buds are exchanged between pigmented and albino Xenopus embryos at stage 32. When a posterior half-bud is grafted orthotopically into an albino host, the resulting adult mosaic shows a distinct boundary between the black and white halves. When the reciprocal graft is done, the adult eye shows no boundary on external view. Histologic sections show a sharply bounded mosaic of pigment retina, but in the white-into-black chimera, the choroid layer outside the white graft was pigmented. Whole eye exchanges at stage 32 pigmented autonomously, showing that new choroidal pigment was not coming from extraocular parts of the host. Observations on orthotopic half-bud grafts at stage 32 were continued over several post-operative weeks. Choroid pigment from the black host fragment appeared to "migrate" in a dorsal-to-ventral direction. At one week, the boundary had moved significantly and by two weeks the white-into-black half-eye mosaic appeared completely pigmented. Grafting anterior half-buds, white-into-black, produced a similar blackening and disappearance of the boundary. "Migration" was observed in 17 of 18 white-into-black half-eye mosaics, while 80–90% of black-into-white cases retained a sharp boundary on external view. It appears that the choroid follows different rules of development from the more orderly "sector" patterns of the pigment retinal epithelium. More work is needed to discover how the pigment "migrates": Is it actually motile? Does it divide and migrate? Does the moving pigment boundary accurately reflect the shifting positions of wild-type cells? Nevertheless, the unusual pattern of choroidal development helps us understand the complex process of organogenesis in the vertebrate eye.

We thank NSF (PCM-77-26987) and the Alfred Sloan Foundation.

Retinotectal patterns and patterns of genetic mosaicism in ploidy chimerae of Xenopus *eye.* R. KEVIN HUNT, BEN G. SZARO, ROBERT TOMPKINS, AND DANA REINSCHMIDT.

Frog retina is a model system for study of organ morphogensis and development of nerve patterns. We have prepared genetic mosaics of *Xenopus* retina by microsurgically grafting small wedges of eye-bud tissue at stages 31–36 from pigmented tetraploid donors into diploid host embryos homozygous for the albino (alb^P) mutation. These orthotopic grafts ranged in size from small (30°) sectors of eye-bud to half-buds. Two batches of such chimerae ($N = 71$, $N = 76$) were observed closely during healing: the 4n tissue (Tompkins *et al.*, 1980, *Biol. Bull.*, **159**: 455) acquired its black phenotype during the late 30s stages, and the two fragments healed together leaving a contiguous but variable "arc" of marked cells on the ciliary marginal growth ring, from which annuli of new cells are added to the periphery of the eye throughout larval growth. Pigment retinal polyclones, in the 25 individuals reared through metamorphosis, showed "radial line" boundaries; the pigmented 4n cells were contiguous and occupied a "sector" radiating out from the optic disc to the ciliary margin. The detailed structure of polyclone boundaries, and the nuances of form seen in polyclones growing out from different angular positions, conformed closely to patterns seen in mosaics prepared with other markers. Extracellular electrophysiologic recording was used to analyze the visual field projection from the chimeric eye to the contralateral optic tectum. Ten frogs, whose retinal mosaic patterns ranged from small sectors (at 5, 6, 7, 3, 9, or 6 o'clock) to half-and-half patterns, all showed visual projections of normal continuous topography and normal metrics. We conclude that the tetraploid strain offers a powerful marker which is minimally intrusive into retinal growth and retinotectal patterning.

We thank NSF (PCM-79-03827; PCM-77-26987) and the Alfred Sloan Foundation.

A developmental analysis of an unusual homoeotic mutation, proboscipedia, in Drosophila melanogaster. ANNA W. SEITZ, PETER B. MONK, AND THOMAS C. KAUFMAN.

Current theories of pattern regulation in limb development propose that all limb structures proximal to the most distal structure present be represented. The homoeotic mutation proboscipedia of *Drosophila melanogaster* effects labial structures in the adult fly. One allele of this locus, pb^5, causes the transformation of labial structures to prothoracic leg structures in which femoral and tarsal, but not tibial segments are present. The above theory leads us to expect that presumptive tibial cells existed at one point during limb formation, but that these cells then died either after the limb tissue was competent to replace them or as fast as they were formed. To test this hypothesis the labial discs from pb^5 homozygous larvae were examined for cell death. No cell death was observed in discs of early third instar larvae. Some cell death was seen in late third instar larvae, but this did not correlate with the expected location of presumptive tibial tissue. When the morphology of the mutated labial discs was carefully examined, the left and right discs of a pair were found to differ and the morphology of neither disc resembled that of a leg disc. Mapping of the mutated disc is in progress and will clarify the observed morphology. The absence of cell death in the expected areas could be explained by the folding of the mutated disc, such that the observed cell death is tibial cell death. Alternatively, pattern regulation which operates at a very early stage in disc development could give rise to presumptive tibial cells that fail to proliferate. The asymmetry observed in pairs of discs is also manifested in adult structures. This asymmetry could reflect the unusual nature of labial discs, which are known to duplicate themselves in conditions where other discs do not.

This work was supported by Public Health Service grants 5 T32 HDO 7067-04 and T32 HD07098 (A.W.S.), GM-21558 (P.B.M.), SO5 RR7031, and RO1 GM24299-01 (T.C.K.). We would like to thank Brooke Kirby for her interest and encouragement in this project.

Reaction-diffusion models of morphogenesis: an application to pattern formation in Xenopus *retina.* SARAH A. SHOAF, KEVIN CONWAY, AND R. KEVIN HUNT.

We present reaction-diffusion model calculations for pattern formation on a disk (representing a wide variety of embryonic anlagen including frog eyes), in order to examine the sensitivity of patterns to changes in initial conditions and perturbations in the geometry of the morphogen-producing space. The models of Kauffman, Shymko, and Trabert and of Gierer and Meinhardt were tested: Analysis of the linearized equations produced appropriate parameters and disk sizes for pattern growth. A computer-implemented finite element method was used to solve the non-linear model equations reiteratively. For the Gierer-Meinhardt model, initial activation (varying in size over two orders of magnitude) of one point on the disk's edge was sufficient to generate the primary gradient. Various parts of the disk were removed (remaining only as diffusible space) from the morphogen-producing cycle to investigate the effects of cells dropping out of the cycle due to cell death and malfunction (single point removed) or differentiation (center removed), as occur in *Xenopus* eye-bud. The resulting patterns had the same general shape and amplitude as normal gradients. Nor did a 2-fold increase in disk size affect the pattern-generating ability of the model. Disk fragments bearing their primary gradient patterns were fused (with gradients in opposite directions, but each parallel to the fusion line). The resulting patterns generated by the model showed many similarities to results of compound eye experiments in *Xenopus*, including both regulative and mosaic patterns. We conclude that the Gierer-Meinhardt model is remarkably stable subject to a wide range of perturbations in the diffusible space, thus allowing it to cope with normal biological variability, and offering an exciting range of possibilities for reaction-diffusion models as mechanisms underlying the spatial patterns of tissue structures.

We thank NSF (PCM-77-26987) and Dean's Fund of Johns Hopkins University.

Application of a polyploid marker to clonal analysis in Xenopus *eye.* ROBERT TOMPKINS, DANA REINSCHMIDT, KEVIN CONWAY, AND R. KEVIN HUNT.

Clonal analysis of *Xenopus* eye development has been undertaken by producing chimeric eyes, at embryonic stages, of normal tissues and tissues marked with the periodic albino (alb^P) or anucleolate (1-nu) mutant. Analysis of the pattern of marked cells in the resulting larval and adult eye facilitates understanding the growth of the eye and can be correlated with the generation of positional information in the chimeric retina, as revealed by its retinotectal map in a normal tectum. Limitations in the above markers led us to develop a new marker, tetraploidy (4 n). Tetraploid frogs, *X. laevis*, were produced by suppressing first cleavage in normal embryos. The low yield of 4n embryos and the high incidence of abnormalities do not allow direct use of pressed embryos; however, selection of tetraploids by karyotypic and cell-size analysis permitted isolation of fertile tetraploid animals. The yield was one in 250,000 eggs pressed. The selected animals differ from tetraploid amphibia produced by other methods in that they produce eggs larger than the expected doubling of diploid volume, and the cell size of 4n offspring remain unexpectedly large (in retina and brain as well as many other tissues), at least through early larval stages. These large cells—as well as nuclear size, DNA content, and nucleolar number—facilitate the identification of the orgins of individual cells in chimeric eyes as well as other experimental procedures (Hunt *et al.*, 1980, *Biol. Bull.*, **159**: 454). About 150 half-eye replacements and orthotopic wedge-grafts were performed at stages 31–36. Preliminary analysis of albino (diploid)/4n (pigmented) chimeric eyes showed that 4n tissues neither overgrow nor undergrow relative to diploid tissues. Our results confirm findings using other markers; and the large cell size of this easily analyzed marker should make the 4n strain attractive to vertebrate neurophysiologists.

Support was provided by NSF (PCM-79-03827; PCM-77-26987) and the Alfred Sloan Foundation.

ECOLOGY

Food preferences and population dynamics of the amphipod Talorchestia longicornis. GLORIA ALLENDE AND NANCY DISE.

Two distinct populations of the amphipod *Talorchestia longicornis* were found grazing at night on a Massachusetts salt marsh, one along the shoreline in piles of *Zostera marina*, *Ascophyllum nodosum*, and *Fucus vesiculosis*, and another on a blue-green algal mat on the lee side of the dunes. Feeding experiments in divided petri dishes revealed the algal mat population preferred blue-green algae and *Enteromorpha* sp. over two species of sulfur bacteria also found on the algal mat. Both populations preferred *Ascophyllum* over blue-green algae and *Zostera,* although the mat population showed a more pronounced difference in preferences

The amphipods on the algal mat were smaller than the amphipods found along the shore; mixing of the two populations may be prevented by ecological or physical barriers. A mark-recapture study of the algal mat population confirmed that the amphipods are highly mobile within their range, and allowed a late summer population estimate of approximately 10,000 adults.

Microbial ecology of algal extracellular products: the specificity of alga-bacterial interactions. WAYNE H. BELL.

Kinetics analyses of utilization of ^{14}C-labeled algal extracellular products (EP) were performed upon a steady-state bacterial population maintained in continuous culture with the diatom, *Skeletonema costatum.* The culture system selected for development of bacteria adapted well to this alga. Adaptation was evidenced by evolution of a kinetics pattern indicating that both uptake and metabolism of ^{14}C-labeled *S. costatum* EP were limited by the same substrate or substrates. Labeled EP from two other algal species, *Thalassiosira pseudonana* and *Dunaliella tertiolecta,* produced kinetics patterns inconsistent with bacterial adaptation to these algae; such patterns included diffusion-limited transport and lines for uptake and respiration that failed to intersect at a common X-intercept. Nevertheless, the absolute rates of EP utilization were similar no matter what the source of the products. Competitive inhibition analyses with unlabeled algal culture filtrates indicated that the major components of the EP pools of *S. costatum* and *T. pseudonana* are chemically similar and differ considerably from those from *D. tertiolecta.* Thus, although rapid uptake of *T. pseudonana* EP by bacteria adapted to *S. costatum* can be explained by the similarities of EP pools of these two algae, rapid uptake of *D. tertiolecta* EP cannot be so explained. The results indicate that bacteria adapted to a given alga-mediated environment retain sufficient metabolic diversity to rapidly assimilate products from other algal species. Stimulation of bacterial activity by an algal bloom does not restrict bacterial metabolism to the principle components of the available EP pool, but enhances the microbial population's ability to rapidly utilize soluble organic compounds from other sources.

The author is grateful to the Steps Toward Independence Program of the Marine Biological Laboratory and to Hamilton College for support of this research.

Studies of methanogenic bacteria from intestinal tracts of marine fishes. HELMUT BRANDL, J. R. PATEREK, FRANCESCA MOLLURA, C. D. TAYLOR, AND E. P. GREENBERG.

The intestinal contents of five scup (*Stenotomus chrysops*) and one smooth dogfish (*Mustelus canis*) were examined with respect to populations of methanogenic bacteria. Oxygen levels in the intestines of scup were less than 1 μM, the limit of detection of implanted oxygen-microelectrodes. This suggested that scup intestines may serve as anaerobic habitats for methanogenic bacteria. Samples of intestinal contents were serially diluted in an enrichment medium and incubated under an atmosphere of H_2 and CO_2 (80:20). The Hungate anaerobic tube technique was employed in order to avoid contact of samples with oxygen After 3–5 days negative pressure had developed within the enrichment tubes, indicating consumption of H_2 and/or CO_2. However, at this time methane could not be detected by gas chromatography. Since sulfate-reducing bacteria were found at densities of at least 10^9/ml in intestinal contents from both *S. chrysops* and *M. canis,* these bacteria may have consumed H_2 in the enrichments for methanogenic bacteria. After an incubation period of 20 days, methane was detected in the atmosphere above cultures from each of the fish, indicating the presence of methanogenic bacteria. Examination of material from enrichment-tube cultures by epifluorescence microscopy revealed three different morphological cell types that exhibited the characteristic fluorescence of methanogenic bacteria: a *Methanococcus*-type and a large and a small *Methanobacterium*-type. Apparently, methanogenic and sulfate-reducing bacteria coexist within intestines of certain fishes.

This research was supported in part by grants from NASA (NAGW-72) and the Foundation of Microbiology.

Distribution and migratory behavior of Ilyanassa obsoleta *in Barnstable Harbor.* G. A. BRENCHLEY.

The distribution and migratory behavior of the mudsnail, *Ilyanassa* (*Nassarius*) *obsoleta,* was monitored at four sites in order to evaluate the snail's role in creating distributional heterogeneity of infauna in Barnstable Harbor, Mass. Millions of adult snails (16–22 mm) immigrating from the subtidal in the spring onto muddy, *Zostera*-free areas at Huckins Island and Calves Pasture Point became widely distributed in moderately high density (500 per m^2). This pattern continued throughout the summer. In contrast, *Zostera* beds near low water containing the introduced periwinkle, *Littorina littorea,* acted as migration barriers to the mudsnails, concentrating them into dense (1000+ per m^2) patches which maintained their integrity as migrating swarms throughout the summer. By late June, about one million snails had entered between two extensive *Zostera* beds on the sandflat off Indian Trail and were proceeding toward the marsh. Most reproductive individuals (16–22 mm) then moved east at 8–10 m per day toward Bone Hill Road, alternating between two *L. littorea* habitats: *Spartina* in the high, and *Zostera* in the low intertidal zone. Migration continued after the reproductive period, possibly related to the abundance of diatoms, sulfur bacteria, and currents. In the laboratory, *I. obsoleta* would not lay eggs in the presence of *L. littorea;* the latter dislodged, but did not ingest, mudsnail egg masses. Smaller individuals (8–14 mm) remained at the marsh edge near the initial point of contact. They expanded into the *Spartina* only when *L. littorea* were rare or manually removed. Another population of small snails (8–18 mm) off Indian Trail remained over the study period within 4 m of the marsh edge. Patterns of infaunal distribution and abundance correlate with abundances of *I. obsoleta:* patchiness was most evident on the sandflat containing migratory snails. Thus the structure of the benthic community is influenced by the presence of *I. obsoleta* and *L. littorea.*

Supported by a Steps Towards Independence Fellowship.

Symbiosis of chemoautotrophic bacteria and marine invertebrates. COLLEEN M. CAVANAUGH.

Transmission electron microscopic (TEM) examination and analysis of lipopolysaccharide indicate that procaryotic cells make up the bulk of the trophosome tissue in a newly discovered species of *Vestimentifera* found near deep sea hydrothermal vents. High activities of enzymes characteristic of sulfide oxidation and of CO_2 fixation have been measured in the trophosome tissue of this species (H. Felbeck, Scripps Institution of Oceanography, pers. comm.). Thus trophosome procaryotic cells may provide an internal chemoautotrophic source of nutrition to these large tubeworms, which lack a mouth and a gut. Based on these studies, the possibility that chemoautotrophic bacteria are symbionts in other marine invertebrates was examined. Tests for ribulose-1,5-bisphosphate carboxylase activity were positive in *Solemya velum,* an Atlantic coast bivalve known to have a very small gut and to live in sulfide-rich sediments. The enzyme activity appears to be located in the gills and/or mantle cavity. Initial examination of *Solemya* gill tissue with TEM indicates the presence of membrane-bound procaryotic cells which appear to be intracellular. Studies are in progress to determine the potential chemoautotrophy of the procaryotes found in *Solemya* and their relationship to the animal. Similar procaryotic inclusions can be seen with TEM in the gill tissue of a new species of large white clam found at the deep sea vents. The possible contribution by chemoautotrophic symbionts to the nutrition of these animals remains to be assessed. This new type of symbiosis may be important to some marine invertebrates inhabiting sulfide-rich environments.

This research was supported in part by the National Science Foundation grant PCM 79-06638 and the M.B.L. Microbial Ecology Course.

The predatory habits of two lycosid spiders in Great Sippewissett Marsh. MICHELLE CORR AND DAVID HUGHES.

Prey preferences for two lycosid spiders were investigated. *Geolycosa pikei,* found in sand dunes near the algal mat, was observed in laboratory experiments to prey upon *Talorchestia longicornis,* an amphipod found in high densities around the algal mats. *Geolycosa,* 13 mm in body length, showed size preference toward small *Talorchestia,* approximately 5 mm in length. *Lycosa hellue,* found in the high marsh near the strand line, was placed in cages in the field with known numbers of three types of prey: *Orchestia grillus,* an amphipod; *Melampus bidentatus,* a snail; and *Philoscia vittata,* an isopod. Of the three, isopod mortality was highest, but differences in mortality between experimental and control cages with prey only were not statistically significant. Amphipods and *Melampus* may have been too large for the *Lycosa* to prey upon. More replications, with smaller amphipods and snails, are necessary to form more substantial conclusions about this predator (*Lycosa*)-prey interaction.

The relationship between diet and growth rate of the grass shrimp Palaemonetes pugio. Randy Chambers and Anthony Pires.

The effect of diet on the growth of juvenile grass shrimp, *Palaemonetes pugio,* was investigated. The laboratory experiment was a Latin square design, with five shrimp monitored for each of five diets (mussel mantle, control and nitrogen-enriched sediments, and two algal species, *Enteromorpha intestinalis* and *Gracilaria verrucosa*). Growth on each diet after 20 days was recorded as percent increase in total body length. Only the mussel diet yielded growth comparable to field population growth, averaging a 57% length increase (the largest on any experimental diet) over the course of the study. No significant differences ($P = 0.05$) in growth were shown between sediment diets (control, 31%; nitrogen-enriched, 24%), although growth on an *Enteromorpha* diet (25%) was greater than growth on *Gracilaria* (11%). Length increases were correlated with nitrogen content and palatability of the foods. These results suggest that in the field, *Palaemonetes pugio* must find concentrated protein sources (for instance, meiofauna or food items scavenged from other predators) that can be translated into fast growth.

Mechanism of heavy metal inhibition of amino acid transport in the intestine of marine fishes. A. Farmanfarmaian, Robin Socci, and Thomas Polidore.

Heavy metals, such as cadmium and mercury, are released from various pollution sources into coastal waters and sediments. Marine fish accumulate heavy metals via food. Small invertebrates, such as annelid worms and clams, which are important sources of food for demersal fishes, have been shown to contain high levels (10–30 ppm) of heavy metals. The effect of such toxicants, released from food in the gastrointestinal canals of fishes, are not known. We have started a project examining the effects of heavy metals ($CdCl_2$, CH_3HgCl, and $HgCl_2$) on digestive-absorptive functions in the intestines of several marine fishes.

The absorption of ^{14}C-labelled L-leucine from buffered fish Ringer at 20°C *in vivo* and *in vitro* was measured in the presence and absence of heavy metals at three concentrations (2–30 ppm). In toadfish *in vivo* experiments $CdCl_2$ and CH_3HgCl had no significant effect but $HgCl_2$ inhibited the absorption rate by 57 and 80% at approximately 10 and 20 ppm of mercury respectively, after 10 min of incubation. In sea robin *in vivo* experiments intestinal tissues were incubated for 10 min. The uptake of L-leucine was not affected by CH_3HgCl but in the presence of $HgCl_2$ 21 and 44% inhibition was observed at the respective concentrations mentioned. Similar levels of inhibition were recorded for winter flounder when tissues were exposed to $HgCl_2$. The oxygen uptake of the tissue, although inhibited by high levels of $HgCl_2$, is not affected as drastically as amino acid uptake during a 10 min incubation. This indicates that Hg^{2+} inhibition involves a direct binding to the intestinal membrane transport mechanism and does not lower amino acid uptake secondarily via reduction in cellular energy production. This view is further supported by the observation that CH_3HgCl, in which mercury has a single positive charge, does not cause appreciable inhibition of leucine transport but reduces oxygen uptake significantly.

Mapping vegetation and topography of Great Sippewissett Salt Marsh, Mass. Amy Friedlander, Francis Bowles, Judith Gale, and Bruce Peterson.

Great Sippewissett Salt Marsh on Cape Cod is a small pocket marsh of approximately 50 hectares. Mapping field work was conducted in the field seasons of 1979 and 1980. The resulting map is to serve three functions: to provide a detailed vegetation map that can be used as a baseline for comparison in the future, to provide a finely contoured relief map that will allow estimates of water volume in the marsh at different stages in the tidal cycle, and to determine the area covered by each major grass species, and the extent to which these areas are exposed or flooded at different tide heights.

Approximately 4000 data points of elevation and vegetation were taken at borders of vegetation types and/or creeks. Elevations were determined by differential leveling, with a level and rod and metric tape. The elevations are referenced to U.S. Geological Survey benchmarks, which provides a baseline datum of mean low water.

The data have been used to determine location and elevation of sites used in peat percolation, litter decomposition, and fertilized plot studies. They have also been used to determine the elevation of a tide gauge, and in studies of slope of the water surface, which have shown that in ebbing and flooding tide there is a maximum slope of as much as 30 cm from the upper reaches of the marsh to Buzzards Bay.

Anticipated uses include analyzing present vegetation patterns and possible future changes, looking at the structure of the barrier beach and subsequent changes in its shape by dune migration or erosion, and calculating volumes of water held in the marsh at various tide heights.

We thank Ian Bowles, Benedicte Misner, Carol Nilson, and Susan Pilling for their assistance in the field. This work was supported by the National Science Foundation under grant DEB 78-03557.

The feasibility of seaweed aquaculture in the Great Sippewissett salt marsh. RODNEY M. FUJITA.

Salt marsh tidal creeks provide certain advantages for the cultivation of marine organisms. High natural concentrations of inorganic nutrients and water exchange due to tidal currents obviate the need for commercial fertilizers and artificial water exchange in the cultivation of marine macroalgae. *Gracilaria verrucosa* and *Chondrus crispus*, red algae valuable for their phycocolloid content and potential energy yield through bioconversion to methane, were grown in floating cage culture at two sites in the Great Sippewissett salt marsh: in a creek draining an area which had been fertilized for 1 year, and in an unfertilized control creek.

During July, *Gracilaria* in the control creek grew at a rate ($10\% \cdot \text{day}^{-1}$) lower than but comparable to those achieved in intensive tank culture, but did not grow well in the fertilized creek. *Chondrus* failed to grow at either site. During August, *Gracilaria* in the control creek grew at a rate of $18\% \cdot \text{day}^{-1}$ before becoming infested with tunicates. Since the cages in the fertilized creek quickly became covered with *Enteromorpha*, an epiphytic green alga, it was thought that water exchange and light intensity limited growth. These results suggest that fouling will be a major problem for aquaculture in naturally eutrophic marsh environments.

Two possible methods for controlling epiphytes were investigated. *Gracilaria* was grown in the control creek and exposed to the fertilized creek every 10 days for 40 hr. This treatment eliminated *Enteromorpha*, while growth rate remained comparable to that of untreated controls. The ability of various marsh animals to selectively graze *Enteromorpha* was investigated in the laboratory. It was found that all animals tested preferred *Enteromorpha* to *Gracilaria*, and thus have potential as biological control organisms.

Statistical mechanics of geomagnetic orientation in sediment bacteria. MICHAEL K. GILSON AND AD. J. KALMIJN.

Last year we reported on time-of-transit experiments in which magnetically orienting bacteria crossed a 1-mm stretch in the direction of a uniform magnetic field. The bacteria were found to behave as tiny self-propelled compass needles subject both to magnetic field alignment and to the randomizing effect of thermal agitation. In strong fields, magnetic bacteria are held in tight alignment; in weaker fields, their swimming paths meander more and transit times are greater. Paul Langevin derived an expression for the distribution of orientation in an ensemble of free-moving dipole particles as a function of ambient field strength. His theory becomes applicable to our experiments when bacterial migration is analyzed as a sequence of short steps during each of which the cell swims in a direction randomly selected from the Langevin distribution. The duration of each step, Δt, is actually a time constant of the cell's loss of directionality due to thermal agitation. By thus treating the migration as a process of random walk with drift, we are able to predict the mean and variance of the time of transit across a 1-mm stretch. The behavior of the model depends on three parameters: the randomization time Δt, the cell's intrinsic dipole moment m, and the speed of propulsion V. We use nonlinear regression analysis to estimate these parameters and to fit the behavior of the model to that of the bacteria. We also determine the goodness of fit of the model in its entirety, and the approximate confidence limits of the parameter estimates. The estimated randomization times are in accord with preliminary calculations of rotational diffusion rates. The dipole strengths agree well with those expected on the basis of the number and size range of the bacteria's intracellular magnetite crystals. Our values are slightly lower due to the inevitable impurities and imperfections in alignment of the crystals, and to additional agitation resulting from swimming movements. In short, the dipole moments direct the bacteria magnetically despite thermal agitation and swimming noise. As statistical mechanics suffice to explain the orientation of magnetic bacteria, there is no need to invoke an active orientation mechanism.

(Kalmijn's project on electric and magnetic detection operates under the auspices of the Office of Naval Research, Oceanic Biology Program, N00014-79-C-0071.)

Larval settlement on microbial films: A model system. STEPHEN GRAHAM, DAVID KIRCHMAN, AND RALPH MITCHELL.

We have found a tube-forming polychaete, *Janua* (*Dexiospira*) *brasiliensis* (Sedentaria: Spirorbidae) useful in studies of larval settlement on microbial films. Spirorbid and serpulid worms are important marine fouling organisms.

Janua is a good model animal for several reasons. It is small (2–3 mm) and hermaphroditic. It is abundant on a variety of surfaces, especially on *Zostera* (eelgrass) from Woods Hole to Buzzards Bay, MA. At least 50% of a typical population is brooding eggs at any one time. Larvae are readily obtained from external brood sacs located in the operculum, and most (60%) settle and metamorphose within 2 hr. One limitation we encountered in Woods Hole was that fertility rates declined over the summer, possibly associated with seasonal temperature. By comparison, *Janua* sampled off Long Beach, CA, produced ample numbers of larvae (65% of adults brooded eggs).

We demonstrated that larvae prefer to settle on surfaces coated with microbial films. Settlement and metamorphosis does not occur on films of the diatom *Nitzchia*. In the absence of microbial films, *Janua* larvae failed to settle. Gama-aminobutyric acid did not induce settling. Uni-bacterial cultures of *Pseudomonas marina* and a few other specific bacteria induced settlement and metamorphosis, but most of the marine bacteria tested lacked this ability.

We attempted to identify the metamorphic trigger produced by the bacteria. Chloramphenicol did not block the triggering action of the bacteria, providing evidence that protein synthesis is not essential for settlement. Formalin-treated films did not inhibit metamorphosis, indicating that viable cells are not required. No metamorphosis was detected on surface films prepared from either cell wall components or intracellular material produced by lysis of the bacteria. The metamorphic trigger may be associated with either extracellular polysaccharides or other loosely-bound bacterial polymers.

This work was supported in part by NOAA grant NA79AA-D-00091 and ONR contract N00014-76-C-0042 to Harvard University.

The relationship between organic and nitrogen content of marsh sediments and feeding rates in Uca pugnax. ERICH F. HORGAN AND MARGARET S. RACE.

The ability of the deposit-feeding crab *Uca pugnax* to alter its feeding rate in response to sediments varying in organic and nitrogen content (% total dry weight) was investigated in the laboratory. Crabs starved for one day were observed feeding on six different sediment types: the first three sediments contained 45% organic matter with nitrogen values ranging from 1.26 to 1.35%, while the second three sediments contained 20% organic matter with nitrogen values ranging from 0.38 to 0.63%. Under both organic content regimes, feeding rates were inversely correlated with the nitrogen content of the sediments: as nitrogen content increased, feeding rates significantly decreased. These results suggest a relationship between feeding behavior and nutrient turnover in benthic systems.

We extend thanks to Ivan Valiela and John Teal for their helpful ideas, and appreciation to the Founders of the Marine Biological Laboratory Scholarship Fund in assisting in making this research possible.

Preferred food sources and the limitation of local distributions of the isopod crustacean Philoscia vittata. DAVID HUGHES AND MICHAEL USEM.

Food preference of the isopod *Philoscia vittata*, testing *Spartina* plant parts and detrital material, was determined in laboratory experiments. Preference, measured by the observed production of fecal pellets in petri plates containing agar suspensions of the foods, was marked for detrital material from two sources. No significant difference between preference for *S. patens* or *S. alterniflora* detritus was found, suggesting that food choice does not determine distribution of the isopod. In the field, isopods were caged in areas of the marsh above and below areas of known distribution to test the effects of water immersion and dessication. Survivorship after 8 hr under water was 100%; survivorship in wooded areas bordering the marsh was variable, increasing with the amount of grass cover. Dessication may limit the upper distribution of the isopod in the marsh, but no lower limit factors were identified.

Tidal water exchanges between Great Sippewissett Salt Marsh and Buzzards Bay.

DAVID W. JUERS, FRANCIS P. BOWLES, AND BRUCE J. PETERSON.

The construction of a sulfur budget for Great Sippewissett Marsh requires accurate estimates of water volume exchanges between the marsh and Buzzards Bay. These estimates are derived from 24-hr continuous records of tide height and current velocity made during monthly samplings. To examine the patterns of water transport implied by these data, ebb and flood volumes were measured on four tides which had amplitudes ranging from 0.85 to 1.25 m.

Throughout each tidal cycle, water velocity measurements were made with an EMF-type current meter, at 20 cm depth intervals every 4 m across the channel behind the inlet to the marsh. The area at each location was computed from depth measurements taken during the tidal cycle. A second current meter, connected to a data logging system, continuously recorded water velocity 30 cm from the surface, at the first transect location. Flow rates at each location were calculated and summed to yield total water flow.

Integrated over time, these measured rates yielded tidal exchange volumes which varied from 85,000 to 220,000 m^3 for flood tides, and 110,000 to 160,000 m^3 for ebb tides.

Predictions for flood tides were obtained by fitting a modified logistic equation to the cumulative volume curves. Further conditioning the equation with factors relating peak water velocity to tidal amplitude, and tidal amplitude to the length of the flood tide phase, yielded predictions of total volume that differed from those measured by only 3–8%. For any time during the ebb, the amount of water leaving the marsh was found to be a linear function of the water volume input on the flood tide.

This work was supported by NSF DEB 78-03557.

Bacterial epiphytes on Zostera marina *surfaces.*

D. L. KIRCHMAN, L. MAZZELLA, R. MITCHELL, AND R. S. ALBERTE.

A complex microbial community, comprised of bacteria, diatoms, and other microorganisms, is present on *Zostera marina* L. (eelgrass) leaf surfaces. To increase our understanding of the relationships between the epiphytic community and *Zostera,* we tested whether bacteria on the leaf surface obtain organic carbon from the leaves to support their production. Individual *Zostera* leaves were placed with their tips in dark chambers and their bases in illuminated chambers. Sodium [^{14}C] bicarbonate was injected into the illuminated chambers, allowing radioactivity to appear in the dark chambers only by translocation through the *Zostera.* Epiphytes were removed from the *Zostera* leaf surface with a razor blade with no detectable damage to leaves as determined by microscopic examination. Nearly 20% of the total translocated radiolabel was recovered in the dark chamber epiphytes. The absolute amount of radioactivity was approximately three times background levels. Radioactivity in the dark chamber epiphytes can only be due to epiphytic bacterial uptake of organic carbon from the *Zostera,* since dark $^{14}CO_2$ fixation is extremely low.

In order to eliminate possible accumulation of radiolabel independent of epiphytes on the leaf surface in the dark, epiphytes were removed from leaf surfaces, and then the leaves were incubated as described above. The amount of radiolabel associated with the epiphytes in the dark chamber dropped to only 2% compared with 20% in the previous experiments in which epiphytes were present. This amount was barely above background levels.

An estimate was made of epiphytic bacterial production specifically supported by the *Zostera* tissue. Bacterial production per cell was estimated to be $(1.3–13) \times 10^{-7}$ $\mu gC \cdot hr^{-1}$, based on literature values. Bacterial numbers on *Zostera* surfaces were approximately 10^7 cm^{-2}. Total bacterial production was then calculated to be 1.3 to 13 $\mu gC \cdot hr^{-1} \cdot cm^{-2}$. Since the measured carbon fixation rate of *Zostera* was 3.5 $\mu gC \cdot hr^{-1} \cdot cm^{-2}$, and since recovery of label from *Zostera* photosynthesis in the epiphytes was 20% of that translocated, it was calculated that 0.7 $\mu gC \cdot hr^{-1} \cdot cm^{-2}$ was available to the epiphytes from the *Zostera.* Therefore, 5–54% of total bacterial production could be supported by carbon fixed by *Zostera* alone. These calculations and the experiments described above suggest that a significant amount of epiphytic bacterial production is supported directly by *Zostera* photosynthesis.

This work was supported in part by NOAA grant NA79AA-D-0091, ONR contract N00014-76-C-0042, and NSF grants PCM 79-06638 and PCM 78-10535.

Contextual relationships in food webs involving meiofauna. JOHN J. LEE AND MONICA J. LEE.

Conceptual models of energy flow in shallow water benthic marine food webs involving small animals (microfauna and meiofauna) and microflora have a common weakness. They fail to account for food-quality-related aspects of energy flow. Potential energy in these communities as detritus, or as the substance of bacteria, microphytes, and fungi is always in excess. Gnotobiotic nutritional experiments suggest most successful protozoa and micrometazoa have the potential to optimize their energetic needs by selectively consuming microfloral species. The properties of quality related (or informational) energy flow are not only intrinsic to the molecular constitution of food, but also to molecular processing after consumption and nutritional requirements of consumers.

Enigmatically, most meiofaunal species raised gnotobiotically are fecund on diets of algae with low abundances. It could be argued that nutritional experiments involving one or only a few potential food species are misleading. They present fewer choices than encountered in the "real" world. In the absence of competition the variety of microorganisms available as potential food in any benthic community should satisfy nutritional requirements of small grazing herbivores. We have been testing this hypothesis. Selected species of meiofauna were inoculated into natural assemblages of aufwuchs microflora and detritus (from which all animals had been mechanically removed) and incubated either in the nearby Greater Sippewissett salt marsh or in the lab in tissue culture flasks with nylon windows (3 μm pore size) to permit free passage of sea water.

Of the four animals tested, only *Allogromia laticollaris*, a foraminiferan, steadily increased in all natural assemblages into which it was introduced. Populations of *Chromadorina germanica* (nematode) and *Nitocra typica* (harpacticoid copepod) reproduced vigorously in some natural assemblage contexts but were unable to do so in others. *Rhabditis marina* (nematode) failed to reproduce in any of the nutritional contexts tested. Diatom assemblages are being studied to see if the animals modify the communities in which they grazed.

Supported by NSF grant OCE 79 29485.

The effect of chromium on microbial activity in salt marsh sediments. BRUCE LEIGHTY.

A basis of marsh productivity is the decomposition performed by the microbes of the salt marsh sediments. As the health and performance of these microbes are functions of the environment, the effect of pollution is of great importance. This study was conducted to test the effect of a heavy metal pollutant, chromium, on microbial activity in salt marsh sediments.

Two experimental plots with controls were set up in a stand of uniform *Spartina alterniflora* (short form). The experimental plots were subjected to thrice weekly applications of 10 ppm Cr solutions. Scrape samples from the aerobic surface of the sediment were collected and homogenized in the laboratory. Inoculums were placed in blackened biological-oxygen-demand bottles with 1 cc of sterile substrate and filled with 50% sterile seawater. Oxygen levels in the bottles were monitored every 12 hr for 48 hr.

Exposure to Cr was found to reduce oxygen uptake by 80% after 12 hr. However, when samples were incubated without Cr, oxygen uptake immediately returned to normal, suggesting that exposure to Cr for the length of this study had no long term inhibition on microbial activity.

In tests for metal tolerance, both experimental and control plot microbes were incubated with Cr. It was found that following Cr applications, the microbes from the experimental plots were more tolerant of the presence of Cr and exhibited greater oxygen uptake than the control plot microbes, implying that Cr resistance had developed.

To test if this tolerance was a specific response to Cr, or a general response to metal stress, microbes were also incubated with copper. Experimental plot microbes were also found to be more tolerant of Cu stress than controls.

Interactions between two species of littorines—Littorina littorea *and* L. saxatilis—*along New England shores.* Randa A. Mansour.

Aproximately 110 years ago the snail *Littorina littorea* was introduced to Nova Scotia from Western Europe. *L. littorea* has since spread as far south as Virginia. In New England it has become the most abundant periwinkle on both exposed and sheltered shores. *L. littorea* prefers moist areas and is found at high densities from the low- to mid-intertidal. The native periwinkle, *L. saxatilis*, inhabits the mid- to supra-intertidal, increasing in abundance in the higher tidal zones. The changes that occurred with the introduction of *L. littorea* have not been documented. The purpose of this study was to determine the nature of the interaction between *L. littorea* and *L. saxatilis.*

On 24 June 1980, 18 cages measuring 8 × 8 × 3 cm were attached to the pilings of a private dock near Nobska Point, Cape Cod, Mass. Cages were placed in the supra- and mid-intertidal zones. Ten *L. littorea*, 10 *L. saxatilis*, or 5 animals of each species were assigned to a cage. One cage at each level was empty. On 12 July 1980 snails were removed, growth was measured as lip increments, and the relative food abundance in each cage was determined.

Both species grew significantly more in the mid- than in the supra-intertidal. Intraspecific competition for food limited the growth of *L. littorea* in the supra-intertidal. At the mid-intertidal *L. littorea* depressed the growth of *L. saxatilis.* These results indicate that in the absence of *L. littorea*, *L. saxatilis* would have a broader vertical range.

Cadmium resistance in bacteria from the Great Sippewissett Marsh. F. Mollura.

Plots of *Spartina* in the Great Sippewissett Marsh have been fertilized with commercially-prepared sewage sludge containing substantial amounts of heavy metals. The study addresses the question of whether cadmium (Cd) resistant strains of bacteria have developed, and begins preliminary study regarding mechanism of detoxification.

Isolates of bacteria from the upper centimeter of marsh sediments were obtained from sludge-fertilized and unfertilized plots of *Spartina.* These were tested for ability to grow on Cd^{2+} supplemented and unsupplemented agar media. Two types of populations were grown, one on nutrient-rich Zobell's (2216E) agar and the other on diluted medium (2216E/100). Clones that appeared to be Cd resistant by their ability to grow on 10^{-3} M Cd^{2+} supplemented 2216E were found on the fertilized plots but not on the unfertilized one. More colonies of resistant bacteria were found on the plot fertilized for 6 years than the plot fertilized for < 1 year, suggesting that adaptation and ability to grow in the presence of Cd^{2+} requires time. Bacteria grown on the diluted medium were unable to grow with 10^{-3} M Cd^{2+}, and grew very poorly on 2216E/100 with 10^{-4} M Cd^{2+}. These same bacteria grew very well on 2216E with 10^{-4} M Cd^{2+}. Isolates from 2216E with 10^{-3} M Cd^{2+} were unable to tolerate 10^{-3} M Cd^{2+} concentration on 2216E/100. This suggests that the presence of greater amounts of organic material may provide a more favorable environment for detoxification.

This work was supported in part by grants from NASA (NAGW-72), The Foundation of Microbiology, and Le Moyne College, Syracuse, NY. Gratitude is expressed to Dr. Jeanne S. Poindexter for her assistance with this project.

Control of drilling fluid discharge from petroleum development on Georges Bank. Elizabeth Mullin.

Drilling muds, which are pumped down the bore hole to cool the drill bit, control pressure, and bring cuttings to the surface, may be discharged during drilling for oil and gas on Georges Bank. Studies to predict their fate and effects on marine ecosystems have yielded conflicting and ambiguous results. However, studies do suggest that drilling fluids could be widely dispersed throughout the water column and concentrated in certain depositional areas. There is also evidence that drilling fluid components, which include heavy metals and bactericides, may cause lethal or sublethal effects on marine biota.

Several strategies are available to mitigate possible impacts. Transportation of used muds to shore for land-based recycling or disposal, or reinjection of muds into the rock formation would minimize the potential for contact with marine organisms. Piping or barging of effluent off the continental shelf could reduce risk to the fisheries of Georges Bank, but the volume of muds entering the ocean would not change.

The oil companies favor shunting drilling mud through a pipe into the water column, but the efficacy of this technique is questionable. Shunting is unlikely to localize impacts because of circulation patterns in the region. Controls over discharge rates and dilution of muds before discharge may reduce initial concentrations, but, like shunting, do not reduce the amount of mud discharged on Georges Bank. Physical or chemical treatment of wastes before discharge could provide some additional protection. Altering the composition of muds could reduce the amount of toxic components. However, chronic, low level contamination of Georges Bank may cumulatively or synergistically affect the fisheries.

Food choice and palatability in a salt marsh detritivore, Melampus bidentatus.

CAROL S. RIETSMA.

Detritivores play an important role in decomposition of higher plants by comminuting and assimilating detritus. A number of properties of detritus affect its palatability to detritivores. This research investigated effects of plant species, age, nitrogen content, amino acid content, ferulic acid content, salinity, and pH of the detritus on feeding by the common salt marsh detritivore, *Melampus bidentatus.*

Relative palatability was measured by counting the number of feeding marks left by snails on the surface of agar suspensions of detritus in petri dishes with four compartments. The snails were offered the choice of feeding on either two or four different foods.

Melampus bidentatus preferred to feed on detritus from *Juncus gerardi* over that from *Spartina alterniflora* and *S. patens,* both of which were preferred over *Distichlis spicata.* Natural aging of detritus from *S. alterniflora* in the field reduced its palatability. Irrespective of age, a higher nitrogen content enhanced its palatability, whereas phenylalanine and leucine reduced it. Aspartic acid, glutamic acid, glycine, and betaine had no effect. Ferulic acid, a feeding inhibitor, interacted with salinity and pH to affect the palatability of old detritus. Properties of detritus such as its plant species, age, and nitrogen content, etc., determine whether or not it is consumed by detritivores and thus affect the rate of decomposition of detritus.

This research was supported by an NSF grant DEB 7905127.

Denitrification potentials in a successional sequence of northern hardwood forest stands.

J. P. SCHIMEL, P. A. STEUDLER, J. M. MELILLO, J. G. WARREN, C. M. ZACKS, AND J. D. ABER.

Denitrification potentials were measured using an acetylene technique in four north-central New Hampshire hardwood forest stands—2, 3, 30, and 50 years since clear cutting. The procedure used involved the following steps: (1) short term (less than 2.5 hr) *in situ* incubation of freshly taken, unamended soil in a closed vessel with an atmosphere containing 10% acetylene in nitrogen; (2) periodic sampling of gas in the incubation vessel and storage of the gas samples in Vacutainer tubes; and (3) analysis of gas samples for nitrous oxide concentrations using ^{63}Ni electron-capture gas chromatography. Soil samples were analyzed in the lab for moisture, organic matter, pH, and nutrient concentrations.

The results of our study show that denitrification potential was highest in the forest floor of the 2-year-old stand (560 ng $N_2O\text{-}N \cdot g$ organic matter$^{-1} \cdot$hour^{-1}), intermediate in the forest floors of the 50- and 3-year-old stands (150 and 47 ng $N_2O\text{-}N \cdot g$ organic matter$^{-1} \cdot$hr^{-1}, respectively) and lowest in the 30-year-old stand (5.8 ng $N_2O\text{-}N \cdot g$ organic matter$^{-1} \cdot$hr^{-1}). Potentials in the mineral horizons were comparable to the forest floor except in the 2-year-old stand which gave lower values (180 and 360 ng $N_2O\text{-}N \cdot g$ organic matter$^{-1} \cdot$hr^{-1} for the A and B horizons).

Within stand variability among denitrification potential measurements was high (S.D./mean ~ 1). Correlations of soil nitrate, ammonia, and total inorganic N to denitrification potentials were high ($r^2 > 0.9$) across the successional sequence, but low within each stand ($r^2 < 0.5$).

These findings indicate that northern hardwood forest ecosystems, especially recently cut systems, have the potential for nitrogen loss by denitrification in spite of their high acidity (forest floor pH range 3.5–3.9), and their generally well-drained condition.

Effect of grazing by Uca pugnax *on the microbial population in salt marsh sediment.* ANNETTE SPIES AND FREDRIC LIPSCHULTZ.

The fiddler crab, *Uca pugnax,* is an abundant detritivore in Sippewissett Salt Marsh, Falmouth, Mass. The effects of *Uca* grazing on sediment microbial populations was determined by caging *Uca* on 0.2 m^2/plots, and then counting bacterial numbers by acridine-orange epifluorescence. *Uca* were either enclosed in caged plots at 28 animals·cage^{-1} or excluded entirely. Caged and control plots were established on silty sediments of creek bottoms and mudflats where *Uca* primarily grazes. Small sediment cores were removed at 1, 2, 7, 14, and 21 days and the top 1 cm and subsurface 2 cm sectioned and preserved in 10% formalin. Bacterial counts were done on 10 random fields at 100× magnification.

Bacterial numbers doubled from approximately 2×10^{10} cc^{-1} to 4×10^{10} cc^{-1} within 48 hr in the mudflat exclusion plots compared to controls. This was statistically significant ($P < 0.05$). Numbers remained high for a week but declined to control values by Day 21. Population densities at the creek site also increased rapidly and then declined. Meiofauna were enumerated from the plot sediments after 21 days: As with bacterial numbers, populations of worms, protozoans, polychaetes, and copepods were identical in control and crab inclusion plots. Mudflat exclusion plots, however, had four times as many worms and six times as many protozoans as the controls. This was statistically significant ($P < 0.05$).

Removal of a major predator, *Uca pugnax,* from the sediment surface resulted in rapid increase of bacterial numbers from steady state levels, followed by greatly increased meiofaunal numbers, possibly in response to higher bacterial prey densities and release from crab predation.

Effects of closed-culture competitive interactions on growth of Teredo navalis *larvae.* GREGORY A. TRACEY, CARL J. BERG, AND RUTH D. TURNER.

Larvae of the shipworm *Teredo navalis* were reared in closed culture under experimental conditions designed to distinguish between genetic variation and possible size-dependent competitive interactions. No differences in growth rates, survivorship, or maximum size of the larvae after 14 days of growth were observed for culture densities of 250–1000/l and volumes of 1–3 l. Differential growth rates were observed among larvae obtained from populations divided by sieving into large and small fractions at 90 and 120 μ mean height. In both cases, the growth rates of the smaller fraction were greater than the larger fraction or controls until larvae had achieved a size of 140 μ in height, at which point their size-frequency distributions overlapped. This phenomenon cannot be totally explained by genetic variation, as the size-frequency distributions of large and small larvae, seen as a bimodal peak in the control groups, remained distinct throughout this period. It remains unclear what mechanism of competition might be occurring, or if size-dependent competitive interactions are possible in the natural habitat.

The effect of sewage fertilization on benthic macroinvertebrates in salt marsh creeks. TIMOTHY UYEKI AND WENDY WILTSE.

Species composition and population densities of benthic invertebrates were studied in salt marsh tidal creeks treated with three dosage levels of sewage sludge fertilizer (0.8, 2.5, and 7.5 g $N \cdot m^{-2} \cdot week^{-1}$), urea fertilizer (2.5 g $N \cdot m^{-2} \cdot week^{-1}$), and untreated controls. Oligochaetes and polychaetes predominated, with the greatest number of species occurring in creeks treated with the highest dosage of sewage fertilizer. Densities increased during the spring, reached a maximum in early summer, and decreased dramatically by August under all treatments. The highest density (340,000 m^{-2}) occurred in June at the highest level of sewage fertilization, suggesting that food availability influenced recruitment or survival of benthic invertebrates. Densities below 100 m^{-2} in all creeks in August indicated that intense fish and crab predation depleted populations of all species during summer. Sewage fertilization may increase production of benthic invertebrates in salt marsh creeks, but increased densities were evident only in early summer when predation was not intense.

Rifampin as a selective agent for the isolation and enumeration of Spirochaeta *from salt marsh habitats.* FREDERICK H. WEBER AND E. P. GREENBERG.

Members of the genus *Spirochaeta* from various salt marsh habitats were enumerated and isolated directly using a prereduced complex agar medium containing rifampin as the selective agent. In the absence of rifampin, *Spirochaeta*-type colonies constituted 7% of the total colony-forming units (CFU) from samples of the top cm of sediment from a pan in the Great Sippewisset salt marsh. At a rifampin concentration of 10 μg/ml, *Spirochaeta*-type colonies constituted 56% of the total CFU. At higher rifampin concentrations (30 μg/ml) formation of *Spirochaeta* colonies was inhibited. Thus the medium used in subsequent experiments contained rifampin at 10 μg/ml. Spirochetes were enumerated and a variety of morphological types isolated from the top cm of sediment from several habitats in Great Sippewisset salt marsh including *Spartina* stands, cyanobacterial mats, sandy and muddy creek bottoms, and pan areas. *Spirochaeta*-type CFU generally ranged between 10^4 and 4×10^5/g wet weight of sediment but exceeded 10^6/g wet weight in two samples from pan areas in the Barnstable marsh. Studies of vertical distributions in cores of the top 5 cm of sediment from pan areas and *Spartina* stands revealed that *Spirochaeta*-type CFU were most prevalent in the upper 1–2 cm—generally at least 10-fold more frequent than in deeper sediments. Apparently, the habitats studied provided favorable environments for certain members of the genus *Spirochaeta*.

This research was supported in part by grants from NASA (NAGW-72) and the Foundation of Microbiology.

The effectiveness of National Park Service policies in protecting barrier island ecosystems within the Cape Cod National Seashore. JOHN P. WARGO.

This paper analyzes the effectiveness of National Park Service policies in protecting barrier island ecosystems within the Cape Cod National Seashore, established in 1961. The types and intensities of uses permitted by Park Service policies were analyzed. The impacts of such uses on beach, dune, salt marsh, and mud flat ecosystems were evaluated.

Recent literature indicates that structural development and use of oversand recreational vehicles has had substantial adverse effects on the stability of these dynamic ecosystems.

Legislation permits continued private ownership of improved property within the park boundary, if local governments adopt zoning ordinances meeting Interior Department standards. Development not meeting such standards is subject to federal condemnation. Zoning ordinances adopted by towns have been effective in preventing development of new residential, commercial, and industrial uses on private lands within the Seashore. They have not been effective in preventing the substantial expansion of existing residential uses. Present Park Service policy permits the reconstruction of residential structures on barriar island ecosystems if the homes are destroyed by storms.

Park Service policy permits the use of recreational vehicles on established routes. In 1979, 30,000 vehicle trips occurred on federal lands within the Seashore and 5000 vehicle trips on town land.

Legislation permits continued town ownership of land within the Seashore. The Park Service has no control over structural development or the use of recreational vehicles on more than 2200 acres of such land.

The Park Service has no method but condemnation to enforce land-use regulations on private land. Enforcement is therefore dependent upon the availability of funds to buy land.

FERTILIZATION AND REPRODUCTION

Change in cyclic nucleotide content during germinal vesicle breakdown in Spisula *oocytes.* OYEWOLE ADEYEMO, HIROKO SHIRAI, AND SAMUEL S. KOIDE.

Spisula solidissima prophase-arrested oocytes can be activated by insemination or application of chemicals. Reports suggest a relationship between germinal vesicle breakdown (GVBD) and cyclic nucleotide content, *e.g.* in *Xenopus laevis*, injection of a regulatory subunit of cAMP-dependent protein kinase induces GVBD while injection of a catalytic subunit inhibits GVBD induced by progesterone. In *Rana pipiens* a reduction in cAMP and cGMP occurs shortly after progesterone application. In the present experiment fluctuation in cyclic nucleotide content during GVBD in *Spisula* was investigated. Oocytes from 5–7 animals were suspended in seawater (0.5 ml packed oocytes/5 ml). GVBD was induced by adding 0.5 ml of 0.5 M KCl or 1/30 dilution dry sperm to 5 ml of oocyte suspension. At appropriate time intervals (0, 1.2, 4, 6, 8, 10, 20 min) oocytes were collected by centrifugation. To the pellet 5 ml of 0.5 M perchloric acid in 25% ethanol (v/v) was added. The mixture was homogenized and centrifuged. The supernatant was neutralized with solid $KHCO_3$, centrifuged, and dried (55C,N_2). Assay buffer (0.5 ml of 0.05 M Tris-HCl, 4 mM EDTA, pH 7.5) was added and the pH adjusted to 7.5. We estimated cGMP and cAMP (four experiments) by radioimmunoassay and the values expressed as pmol/10^6 oocytes. The ratio of cAMP to cGMP content in unstimulated oocytes was 1.7. There was an initial consistent fall in cGMP level (2.8) 1–4 min after stimulation of GVBD, followed by a return to the zero-time level (5.6). A second decrease occurred at 8–10 min or thereafter. No significant change in cAMP level (9.0) throughout GVBD was observed. These findings suggest a probable involvement of cGMP-dependent processes in GVBD in *Spisula* oocytes and further indicate that the two nucleotides have different metabolic pathways.

This investigation was supported in part by the Rockefeller Foundation, Steps to Independence fellowship from M.B.L. (H.S.), a travel grant from Yamada Science Foundation (H.S.), and NICHHD grant R01-HD13184.

Properties of isolated fertilization envelopes from Arbacia punctulata. Lucio Cariello, Jay C. Brown, and Brian Storrie.

Arbacia eggs were fertilized in the presence of 1 mM amino-triazole (ATA), a peroxidase inhibitor, to prevent hardening of the fertilization envelope. Eggs were then passed through a 51 μm Nitex filter to strip raised fertilization envelopes off the eggs. Fertilization envelopes and eggs were separated by repeated settling. Envelopes prepared by this procedure were found to be intact and substantially free from cellular contamination. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of solubilized envelopes revealed that they contain several protein species with molecular weights between 20,000 and 200,000. Phospholipid could not be detected in purified envelope preparations. Lactoperoxidase-catalyzed ^{125}I labeling of intact *Arbacia* eggs resulted in the labeling of a single high molecular weight protein. After fertilization several lower molecular weight components are also labeled. Any or all of these ^{125}I-labeled proteins could be components of the fertilization envelope. Isolated envelopes were stable to treatment with either 1% Triton X-100 or 0.01 M dithiothreitol, but they were completely disrupted by exposure to 2 mM EDTA or 2 mM EGTA for 30 min at room temperature. This suggests that Ca^{2+} plays a key role in stabilizing the fertilization envelope before it is cross-linked by the cortical granule peroxidase. Envelopes could be stabilized by pretreatment with H_2O_2 (0.003%) for 30 min at room temperature. ATA completely blocked this effect. These results suggest that fertilization envelopes contain endogenous peroxidase activity which may be derived from the cortical granules. Pretreatment of isolated fertilization envelopes with Triton X-100 extracts of unfertilized eggs (in the presence of H_2O_2) also stabilized envelopes against EDTA or EGTA. This should provide an assay for the purification of the peroxidase.

Gossypol inhibits motility of Arbacia *sperm.* Mario H. Burgos, Chang Chih-yi, Leonard Nelson, and Sheldon J. Segal.

Gossypol, the principal pigment of cotton seed, has been given to men to suppress fertility. Clinical investigations have not established the basis for the action of this orally administered compound although direct effect of gossypol on fructose utilization by ejaculated human sperm has been observed. Here, we report that gossypol concentrations from 10–100 μM stop motility of sperm of *Arbacia punctulata* within 12 min. Sperm motility was measured by centrifugal-field quantitative evaluation as well as direct microscopic observation. There is a close correlation between the two methods when they are used to evaluate the effect of lower concentrations of gossypol, but with higher concentrations (50 or 100 μM) agglutination of sperm to form clusters interferes with the optical density readings involved in the centrifugal-field method. Trypan blue staining can be used to evaluate alterations in cell membrane permeability. One hr exposure to 50 μM gossypol results in trypan blue absorption by 50% of treated spermatozoa. Trypan blue absorption is not manifested by unfertilized eggs or developing embryos of *Arbacia* exposed to the same concentration of gossypol. The compound does not inhibit ciliary motion of free swimming blastulae or plutei. SEM observations of gossypol-treated sperm reveal a high incidence of alterations in the shape of head, midpiece, and tail. Blebbing of the membrane is seen along the entire length of the spermatozoon. Cell membranes appear to become extremely adhesive, so that in sperm clusters attachment points are randomly established. Efforts to wash gossypol-treated sperm to reinitiate motility have not been successful. Gossypol-treated eggs, subsequently washed, are successfully fertilized by normal sperm. The action of gossypol on gametes appears to be selective. It inhibits the motility of sperm, probably by altering cell membrane permeability, but does not damage eggs or developing embryos.

Oxygen free radical generation by gossypol: a possible mechanism of antifertility action in sea urchin sperm. MICHAEL COBURN, PETER SINSHEIMER, SHELDON SEGAL, MARIO BURGOS AND WALTER TROLL.

Gossypol, a constituent of cottonseed oil, is a potential male antifertility agent. The polyphenolic dialdehyde structure resembles that of pyrogallol (1,2,3-benzenetriol) in containing multiple oxidizable functional groups. Pyrogallol reacts with oxygen to form superoxide and hydrogen peroxide during autoxidation. We hypothesized that gossypol, which also takes up oxygen and is autoxidized, may also generate free radicals.

Gossypol is a highly reactive compound that inhibits the fertilizing capacity of sperm of the sea urchin *Arbacia punctulata.* We tested the possibility that gossypol toxicity to sperm is due to the production of toxic oxygen metabolites by using the enzymes catalase and superoxide dismutase (SOD) to degrade hydrogen peroxide and superoxide, respectively. Gossypol at 20 μM concentrations completely destroyed the capacity of sperm to fertilize eggs. In the presence of catalase or SOD, however, sperm treated with gossypol fertilized eggs normally. This suggests that the action of gossypol is dependent on the generation of hydrogen peroxide and superoxide.

Pyrogallol, at concentrations similar to those of gossypol, also damages sperm fertilizing capacity, and this action of pyrogallol is also inhibited by catalase and SOD. When used together at lower concentrations, the two enzymes protect sperm against damage by gossypol or pyrogallol more completely than either enzyme alone. Boiled enzyme was without effect in preventing such damage.

It thus appears that the spermicidal action of gossypol results from the production of toxic oxygen metabolites. This finding is consistent with the known sensitivity of sea urchin sperm to small amounts of hydrogen peroxide.

We acknowledge grant support from NIH-CA-16060 to W. T. and from the New York University School of Medicine Honors Program to M. C.

Mechanism of nicotinamide action on germinal vesicle breakdown in oocytes of Spisula solidissima. AKIRA MOMII, F. MOMII AND S. S. KOIDE.

Nicotinamide (Nm) at 5 mM inhibits germinal vesicle breakdown (GVBD) of *Spisula* oocytes. By studying Nm action, the molecular mechanism of GVBD can be clarified, as the intracellular NAD^+ level falls drastically after insemination, and Nm raises the intracellular NAD^+ level.

We have shown that Nm is rapidly converted to nicotinic acid in the *Spisula* oocyte, and that trace amounts of Nm are detected after incubation of oocytes with $[^{14}C]$Nm for 3 min. This metabolic conversion is due to an active nicotinamide deamidase system in the *Spisula* oocyte (839 ± 73 nmol·mg protein^{-1}·hr^{-1}). This finding suggests that the agent responsible for the inhibition of GVBD may not be Nm, but rather a metabolite of the deamidase action, namely nicotinic acid or ammonia. Ammonia is not the inhibitor since it activates *Spisula* oocytes. Moreover, nicotinic acid does not affect GVBD, although it is incorporated into oocytes at the same rate as Nm.

In starfish (*Asterias forbesi*) oocyte GVBD is induced by 1-methyladenine, and nicotinamide deamidase activity is low (2 nmol·mg protein^{-1}·hr^{-1}). Nm incorporated into starfish oocytes remains unchanged after 3 min incubation, and the intracellular concentration of Nm was higher in starfish than in *Spisula* oocytes. This finding suggests that intracellular Nm may not be responsible for the inhibition, and that Nm might affect GVBD indirectly via some mechanism involving interaction with the cell membrane.

The present findings support the hypothesis that Nm blocks an NAD^+-splitting enzyme, thereby increasing intracellular NAD^+ levels. This proposition is based on the reports that incubation of oocytes of *Spisula* and sea urchin with Nm results in a significant increase in NAD^+ level. Our result indicates that the concentration of NAD^+ synthesized from administered $[^{14}C]$Nm after 3 hr of incubation can account for less than 0.5% of the reported level. Thus, the rise in intracellular level is probably the result of an inhibition of the NAD^+-splitting enzyme rather than synthesis of NAD^+ from administered Nm.

Supported in part by the Rockefeller Foundation and NICHHD grant RO1-HD13184.

Receptors, drug interactions and calcium in regulation of Arbacia *sperm cell function.* LEONARD NELSON.

Neurochemical control of motility of sperm cells containing both receptors for and enzymes engaged in synthesis and hydrolysis of acetylcholine, presumably operates through modulation of calcium uptake and intracellular distribution. In earlier studies, epinephrine and norepinephrine showed no evidence of affecting cell movement. Now propranolol, a beta-adrenergic blocker, was found to inhibit cell progression at 1 nM/l while causing a moderate increase at 10 μM/l of filtered sea water (FSW). Quinidine, a beta-adrenergic agonist, had only minimal effects by itself. A pronounced interaction between these drugs appeared: in cells pre-incubated in 0.1 mM or 1.0 μM propranolol/l FSW, 1 mM of quinidine depressed motility while 10 μM sharply increased their swimming speed. Propranolol reportedly also counteracts the effect of the cardiac glycoside, ouabain, which is a specific inhibitor of Mg-activated, Na,K-ATPase. Micromolar propranolol increases the motility of cells pretreated with one micromolar ouabain, but with millimolar ouabain, propranolol slowed them down over a range of concentration from 0.1 nM to 1.0 mM. The local anesthetic procaine, which competes for cell surface calcium-binding sites, depressed movement in this study between 10 mM and 10 μM/l FSW. However, in calcium-free artificial sea water (Ca-f-ASW), the same amounts of procaine, after initially depressing, subsequently double the speed over that of the controls. Moreover, sperm spawned in and diluted with Ca-f-ASW showed increases in both speed and duration of swimming. It thus appears that sperm cells are endowed with a number of receptors which may be involved in motility regulation by modulation of calcium transport.

Supported by N.S.F. grant PCM 80-02358.

Tension generation in ovarian wall by 1-methyladenine during starfish spawning. HIROKO SHIRAI AND YASUAKI YOSHIMOTO.

Starfish spawning is induced by 1-methyladenine (1 Ma) produced by gonads under the influence of a peptide hormone. Since ovarian components such as oocytes, follicle cells, and ovarian walls adhere to each other before spawning and since ovarian walls shrink during spawning, release from adhesion within the ovary and contraction of the wall are essential events involved with spawning. To investigate mechanisms of ovarian contraction we measured tension in ovaries of *Asterina pectinifera* with an isometric tension meter having sensitivity of 0.1 mg. "Isolated walls" without oocytes were prepared by incising ovarian fragments. "Jelly" was prepared from spawned eggs by acidification of egg suspension and concentration (1/100 of the intact jelly layer). Intact ovarian fragments (5 mm in length) generated tension after 1 Ma application (10^{-5} M), with a lag time of 15 min (22°C). The value reached a high plateau (mean 45 mg/fragment) 1 hr after 1 Ma application and remained at this level at least 2 hr (designated as "large tension," LT). Discharge of eggs began 20 min after 1 Ma application. Incised ovarian fragments (with oocytes) generated LT (mean 101 mg) with a lag time on 1 Ma application. On the other hand "isolated walls" (without oocytes) did not generate LT on simple application of 1 Ma. However, LT was generated (mean 26 mg) with no lag time on application of "jelly." Moreover "isolated walls" prepared from 1 Ma-treated ovarian fragments retained large tension (mean 27 mg) only under the presence of 1 Ma. The present findings indicate that 1 Ma indirectly induces contraction of ovarian walls, that "jelly" substance can be considered as a direct inducer of ovarian contraction, and that 1 Ma enables ovarian walls to maintain the generated contraction.

This investigation was supported in part by a Steps to Independence fellowship from M.B.L., a research grant from Rockefeller Foundation, and a travel grant from Yamada Science Foundation to H.S.

The toxic effects of vitamin A on sea urchin gametes. PETER SINSHEIMER, MICHAEL COBURN, AND WALTER TROLL.

The block to polyspermy in the sea urchin *Arbacia puntulata* requires the release of H_2O_2 from the egg during fertilization. The release of H_2O_2 from the egg is triggered by entry of the first sperm. The toxic effects of H_2O_2 incapacitate nearby sperm capable of fertilization, thus preventing polyspermy. Two agents which interfere with the elimination and production of H_2O_2 after fertilization, permitting polyspermy, are soy bean tripson inhibitor (SBTI) and catalase. SBTI prevents the formation of H_2O_2 at the egg's surface through its membrane interaction, while the enzyme catalase degrades H_2O_2. We have shown that retinol or vitamin A also cause polyspermy in *Arbacia* eggs. Like SBTI, vitamin A prevents the production of H_2O_2 by the eggs upon fertilization. Both of these compounds also prevent the production of H_2O_2 and superoxides in human leukocytes upon phagocytosis, suggesting a general action on biological membranes. Vitamin A at 0.2 mg/ml was incubated with *Arbacia* eggs, washed, and resuspended in sea water. After fertilization, these eggs were nearly 100% polyspermic. H_2O_2 release, measured colorimetrically, was diminished 80% in eggs treated with vitamin A.

Vitamin A is also toxic to sea urchin sperm at concentrations which caused high levels of polyspermy (0.1 mg/ml). Vitamin A inhibited the ability of sperm to effectively fertilize eggs. This action was inhibited by catalase and superoxide oismutase, which suggests that sperm damage occurred because of the toxic effects of oxygen free radicals. Thus vitamin A induces opposite effects on eggs and on sperm at the time of fertilization. In eggs, vitamin A inhibits the H_2O_2 generating system necessary for an effective block to polyspermy, while in sperm, it causes loss of fertilization capacity through the generation of superoxides and H_2O_2.

We acknowledge grant support from NIH-CA-16060.

Sperm-oocyte interaction in the surf clam Spisula solidissima. ALOYS G. TUMBOH-OERI AND SAMUEL S. KOIDE.

Fertilization is a complex sequence of events that involves species-specific fusion of plasma membranes of the sperm and egg. It is likely that the initial interactions between sperm and egg are mediated by surface receptors that facilitate recognition and binding. To test this hypothesis, a study was undertaken to determine whether there are sperm receptors on the surface of *Spisula solidissima* oocytes. Germinal vesicle breakdown (GVBD) in the oocyte after insemination *in vitro* was used to assay occurrence of fertilization. When normal oocytes were treated with 0.0001% Triton X-100 for 30 min, washed, and inseminated, GVBD did not occur. This suggested that Triton X-100 had removed oocyte surface components responsible for sperm recognition. A supernatant was obtained from detergent-treated oocytes and Triton X-100 removed by treatment with several batches of SM-2 beads. The extract was concentrated and incubated with washed detergent-treated oocytes for 60 min. Approximately 20% of reconstituted oocytes underwent GVBD. In addition, when normal *Spisula* spermatozoa were pre-incubated with the concentrated extract and subsequently used to inseminate normal oocytes, GVBD did not occur. To determine some of the properties of the oocyte receptor extract, the concentrated extract was boiled for 2 min then used to reconstitute detergent-treated oocytes. These oocytes failed to undergo GVBD when inseminated, indicating that the receptor activity is destroyed by heat. Further, reconstitution of detergent-treated oocytes with sea urchin egg surface-membrane extract failed to induce GVBD, showing that the receptors were species-specific. Finally, treatment of oocytes with 100 μg/ml of the lectins ConA, PNA, DBA, RCA-I and SBA prior to insemination did not affect GVBD, whereas the lectin VEA-I reduced GVBD by 50%. This finding suggests that α-L-Fucose is involved in sperm-oocyte interaction in *Spisula*. It can be concluded that receptors present on the surface of *Spisula* oocytes are involved in recognition and binding of sperms. These receptors are heat labile and appear to be species-specific.

Supported in part by The Rockefeller Foundation and NICHHD grant RO1-HD13184.

MICROANATOMY AND MICROTECHNIQUES

Relationships between dendrite and spine neck diameters in freeze-fractured rat hippocampal formation. KRISTEN M. HARRIS.

Theoretical mathematical models have described how dendritic spine necks might provide synaptic input attenuation and isolation, as well as impedence matching to the dendrite. These models require that as the dendrite narrows, so do spine necks. Granule cells from the dentate gyrus of rat hippocampal formation were chosen to study this relationship empirically because their spiny dendrites taper with distance from the cell body.

In Golgi-impregnated granule cells it is possible to see dendritic taper, count many spines, and observe thin spine necks; but difficult to resolve spine neck diameters. Conversely, examination of serial thin sections through spines yields accurate estimates of their neck diameters, but it is difficult to obtain many observations. The freeze-fracture complimentary-replica technique seemed promising because replicas can be viewed at high magnifications with good resolution of many spine neck diameters.

The diameters of cross-fractured spine necks were measured in six adjacent 50 μm regions from the granule cell bodies to the hippocampal fissure. Cross fractures were measured because they appeared more frequently and were similar to profile diameters seen in the same field. The distribution of spine neck diameters was skewed in each of the six regions, with smaller diameters always occurring more frequently. The dendritic diameter was measured at the location of each spine neck. Although this measure is subject to variability in the freeze fracture plane, we have no reason to believe that narrow and wide dendrites will be selectively fractured at different relative depths.

Measurements from 162 spines showed that spine necks narrow as dendrites taper so that the ratio of spine neck diameter to dendrite diameter does not change significantly. Thus, our preliminary results demonstrate a way to analyze many spine necks, and provide an empirical anatomical basis for models relating spine necks to impedance matching.

The author wishes to thank Drs. Thomas Reese and Dennis Landis for their help and encouragement. This project was done during the MBL Neurobiology course, summer of 1980, and supported by IRP-NINCDS.

Characterization of periodic order in the neuroplasmic lattice of Bufo, Loligo *and* Hermissenda *axons by a Fourier analytical technique in scanning transmission electron microscopy (STEM).* ALAN J. HODGE AND WILLIAM J. ADELMAN, JR.

We have previously demonstrated the presence of an ordered neuroplasmic network or lattice in *Loligo* and *Hermissenda* axons using stereoscopy and optical autocorrelation on transmission electron micrographs of relatively thick (0.1–0.5 μm) sections. The lattice consists primarily of neurofilaments and their periodically arrayed side projections (40–45 nm apart) which act as cross-bridges. Neurotubules and other fibrous elements are often included in the lattice. The same type of lattice structure has now been observed in *Bufo* peripheral axons, with the order best preserved in constricted zones associated with Ranvier nodes and Schmidt-Lanterman clefts. The cross-bridge spacing found is also 40–45 nm. This type of structure accounts for axoplasm's gel-like character and anomalously weak optical anisotropy.

The neuroplasmic lattice is also seen in stereomicrographs taken by STEM, a technique suited to analytical methods for objective characterization of video line signals comprising the picture raster. One method involves orientation of one axis (usually longitudinal) of the structure along the line-scanning direction and synchronized recording of the single-line-mode video signal. Inherent noise in single line traces was reduced by averaging numerous (typically 256) single-picture line repeats. Data was analyzed by computer using Fourier methods. Another method, applicable to highly ordered structures such as myelin, involves signal averaging 16–64 successive lines in high resolution picture mode (1000 lines/frame). This is equivalent to integrating the image along the y-axis before Fourier analysis. Myelin and tropomyosin crystals were used as standards.

These techniques were used on longitudinal sections of axons in *Bufo* peripheral nerves and *Hermissenda* connectives. In all cases, the neuroplasmic lattice exhibited an apparent longitudinal spacing of 40–45 nm with indications of a larger unit cell having a n-fold screw axis. It seems clear that this axoplasmic structural order is primarily a property of neurofilaments and associated cross-bridges in the species examined.

Oriented particle assemblies in the plasma membrane of Tetrahymena: *their deployment relative to cell surface topography, cellular morphogenesis, and sensitivity to stimuli.* LINDA HUFNAGEL.

A freeze-fracture ultrastructural analysis of membrane structure in the ciliate, *Tetrahymena,* revealed three different types of particle assemblies: 1) plate-like arrays, 2) paired linear arrays, and 3) hexagonal arrays. The distribution of these arrays relative to cell surface topography and positions of cellular organelles has now been extensively analyzed from freeze-fracture replicas and thin sections. In addition, the surface of *Tetrahymena* has been examined following deep-etching of cells fixed in glutaraldehyde, washed thoroughly with distilled water, and rapidly frozen in Freon 22. Paired linear arrays appear to be restricted to regions where the cell surface undergoes sharp contour changes, such as the edge of the oral cavity and the crests of interkinetal ridges. These arrays thus appear to be important in cytoskeletal-membrane associations required to establish and maintain surface topography. Hexagonal arrays are limited to the floors of the kinetal valleys, near the anterior tip of the cell; they, too, may function in forming and maintaining the specialized architecture of the deeply grooved anterior end of the cell. The morphology and distribution of the plate-like arrays are most consistent with a role in reception of stimuli. For example, surface analysis of deep-etched cells reveals that the plate-like arrays are manifested externally. Furthermore, thin sections demonstrate that the arrays are locations where the cell membrane, underlying inner and outer alveolar membranes, and internal cytoplasm of the cell have a structural continuity not found in other regions of the cell.

Liposome intraocular drug delivery. JUDITH MEGAW, KAREN GARDNER, AND SIDNEY LERMAN.

We report here studies on concanavalin A (Con A)-mediated binding of liposomes to the dogfish ocular lens and delivery of tetracycline to this tissue by this means. Neutral, positively, and negatively charged vesicles composed respectively of phosphatidyl choline (PC) alone, PC and stearylamine, and PC and phosphatidyl serine were tested. Multilamellar vesicles formed in PBS with or without tetracycline were equilibrated 2 hr at room temperature. The vesicles were washed by centrifugation, incubated 2 hr in PBS containing unlabeled or fluorescein-labeled Con A, washed again, and suspended in PBS. Whole lenses were incubated in liposome preparations, washed in PBS, and fixed for fluorescence (FM) or scanning electron microscopy (SEM). *In vivo*, liposomes were introduced by syringe intracamerally into the anterior chamber. (All controls were incubated with PBS.) After 6 hr, the eyes were removed and the tissues examined by FM or fixed for SEM. SEM and FM showed negatively charged vesicles bound to the lens capsule. *In vivo*, some binding to the corneal endothelium was also noted. Neutral liposomes bound equally to the lens capsule, iris, and cornea. Positively charged liposomes did not bind to any tissues examined. Binding of Con A-containing liposomes was abolished by pre-incubating vesicles with Con A inhibitors. Incubations involving tetracycline employed negatively charged liposomes with unlabeled Con A. Delivery was monitored by FM and fluorescence spectroscopy. Following incubation, tetracycline fluorescence was noted in the lens capsule and to a lesser extent in corneal endothelium, but not in other tissues.

This research was funded by NIH grant AG01309.

Crystalline axes of the test and spine of the sea urchin Strongylocentrotus purpuratus—*A new method of analysis.* KAYO OKAZAKI AND RICHARD DILLAMAN.

The sea urchin skeleton is composed of magnesian calcite. It is generally accepted that each spine and coronal plate is a single crystal with the c-axis of the spine parallel to its long axis, and the c-axis of each plate perpendicular to its surface. In contrast to numerous reports on c-axes, little is known of the a-axes because they cannot be visually determined without cleavage directions. We have succeeded in obtaining etch figures exhibiting the rhombohedral cleavage planes of calcite by acetic acid treatment. Also, by exposing etched skeletons to mixtures of M/10 $NaHCO_3$ and M/10 $CaCl_2$, rhombohedral calcite crystals large enough to determine the directions of a-axes were grown epitaxially on the etched surface. In calcite crystals observed along the c-axis, three ridges surrounding the c-axis form 120° angles with one another and a-axes are 30° to the three ridges.

By observing etched and decorated samples under SEM, the following conclusions were drawn: (1) the spine is a single crystal with its c-axis parallel to its morphological long axis, (2) the interambulacral plate is also a single crystal with its c-axis perpendicular to the surface of the plate, (3) the mammelon of the tubercle is a polycrystalline aggregate in both interambulacral and ambulacral plates, (4) the ambulacral plate is an assembly of seven single crystals, (5) the directions of the three a-axes in the plate are nearly parallel to the three edges of the plate, and (6) the direction of the a-axes of the plate have no fixed relationship to those of the attached spine.

Light micrographs of gold-palladium coated, decorated tests showed reflection patterns indicative of the crystalline axes of individual plates due to the functional front-surface mirrors formed.

This work was partially supported by a Hargitt Fellowship to K. O. and NSF Grant PCM 78-22242.

Evaluation of low light level intensifier vidicon detectors for microscopy. GEO. T. REYNOLDS.

Increased interest in applying image intensifier-TV systems to low light level microscopy has resulted in availability of several commercial systems. Evaluation for application to specific problems requires information on dynamic range, resolution, and system noise. Each of these terms is limited in that separately they provide an incomplete description of the response of the system and they are not independent. Evaluation criteria developed for systems used in astronomy and X-ray diffraction studies are applicable in biological applications. The detective quantum efficiency (DQE) compares the signal-to-noise ratio of the output of the device to that of the input signal and thus measures the noise introduced by the system in a measurement. It can be readily measured and used to characterize detector response over a full range of intensity and spatial resolution. The DQE for film, intensifier-silicon vidicon (SIV), and silicon intensified target (SIT) vidicons has been measured.

In addition a method of determining the rms video noise, which limits the sensitivity of TV detectors, has been developed using a calibrated light emitting diode (LED). In this way the rms noise equivalent input has been determined for a Plumbicon, Chalnicon, and three different commercial SIT's. Values ranged from 3.5×10^{-4} to 5.0×10^{-7} foot candles (fc). The equivalent input for an intensifier (gain 10^5)-Plumbicon system was found to be 1.0×10^{-7} fc (the maximum of the LED emission was at 560 nm). Since image tubes with gains above 10^6 are available, limiting sensitivities of 10^{-8} fc, or approximately 10^5 photons per cm^{2}sec, can be realized. Using slow scan TV readout and time integration, higher sensitivity can be achieved.

Supported by DOE Contract EY-76-S-02-3120.

MOLECULAR BIOLOGY AND BIOCHEMISTY

Subcellular localization and characterization of islet hormone-degrading enzymes in anglerfish islet tissue. G. ERIC BAUER, BRYAN D. NOE, MICHAEL N. MORAN, STEPHEN KAHLER, AND JEFFREY B. NEUSTADT.

Evidence was obtained for the presence of insulin- and glucagon-degrading enzymes in subcellular fractions of islet tissue. Islets were homogenized in 0.25 M sucrose; unbroken cells and nuclei were removed by centrifugation. Remaining particulate and cytosolic components then were separated by centrifugation at $142{,}000 \times g$ for 1 hr. Particulates were reconstituted in 0.25 M sucrose and freeze-thawed before assay. Hormone degradation was assayed with tracer concentrations of ^{125}I-insulin (porcine) or ^{125}I-glucagon (bovine-porcine) in sodium acetate-citric acid buffer for 30 min at 37°C. Intact hormone was separated from fragments by adding trichloroacetic acid (TCA) to 5%, and centrifugation. TCA-soluble and precipitable radioactivities were determined by gamma spectrophotometry. When pH optima were studied, insulin- and glucagon-degrading enzyme activities of cytosol were identical (pH 7.3), suggesting that one enzyme is involved, corresponding to "insulin-specific protease" of other tissues. Also, there were acidic enzyme activities (pH 3.4–4.3) in the cytosol as well as in the particulate subcellular fraction. In order to more precisely localize hormone-degrading enzymes, islet tissue homogenates were fractionated into nuclear, lysosome-rich, Golgi-rich, secretion granule, microsomal, and cytosolic subfractions by published procedures. Acidic hormone-degrading enzymes had the highest specific activity in the lysosome subfraction. Also by this procedure, the major "insulin-specific protease" activity (pH 7.3) was found in the cytosol. "Insulin-specific protease" was sensitive to dithiothreitol-reversible thiol-protease inhibition. Acidic enzymatic activity was sensitive to thiol-protease inhibitors, as well as leupeptin, antipain, and especially pepstatin. Hormone-degrading enzymes were relatively insensitive to metallo-protease and serine-protease inhibitors. Further enzyme characterization and purification studies are now in progress.

Support: NIH AM-19223.

Further observations on the phosphorylation of ribosomal proteins after fertilization of Arbacia punctulata *eggs.* D. BALLINGER AND T. HUNT.

We have previously shown that increased labeling of a 31,000 M_r protein of the 40S ribosomal subunit occurs 4–5 min after fertilization of *Arbacia punctulata* eggs pre-loaded with $^{32}PO_4$.

We tested the hypothesis that this phosphorylation increases the probability that the modified subunit will become involved in protein synthesis by analyzing post-mitochondrial supernatants from $^{32}PO_4$-labeled 30-, 70-, 120-, 180-, and 240-min embryos on sucrose gradients, and assaying for the presence of the phosphorylated protein. The specific activity of the phosphorylated 31,000 M_r protein is approximately the same in free ribosomes and polysomes, although accurate quantification is difficult because of the small proportion of polysomes. Thus, it appears that the phosphorylation may be necessary, but not sufficient, for ribosomal activity.

To elucidate the mechanism of this increased labeling we searched for kinase and phosphatase activities in cell-free extracts of eggs and embryos. We could not detect specific kinase activity, but did detect a phosphatase specific for the 31,000 M_r protein in these extracts. We prepared a substrate for the phosphatase assays by incubating embryos for 4 hr in the presence of $^{32}PO_4$, and removing unincorporated label. This substrate was added as tracer to extracts of eggs and embryos. The label in the 31,000 M_r protein is specifically removed by extracts of eggs or 5 min embryos, but not by extracts of older embryos. We believe that the label is removed by a phosphatase activity because it is extremely specific for the phosphorylated protein, and it is inhibited by various phosphatase inhibitors such as NaF, EDTA, and 10–100 mM PO_4. There is apparently a phosphatase specific for the 31,000 M_r protein in eggs and early embryos that is inactivated shortly after fertilization, leading—perhaps in conjunction with increased kinase activity—to the increased labeling of the ribosomal protein.

This work was supported by NIH Training Grant GM 00265.

ADP ribosylation in nuclei isolated from different embryonic stages of Arbacia. REBECCA P. ELLISON.

Adenosine diphosphate ribosylation is a ubiquitous enzyme-dependent reaction in which the ADPR moiety of NAD^+ is covalently attached to a protein acceptor. The effect of the reaction depends on the cellular function of the acceptor protein. In eucaryotes, the enzyme reaction takes place on chromatin, and various chromosomal proteins, including histones, are modified.

Nuclei were isolated from five *Arbacia* developmental stages and incubated with $^3H\cdot NAD^+$ under various conditions. Incubation of 3 mg/ml protein in 0.1 mM NAD^+ and 5 mM $MgCl_2$ at pH 6.8 and 15°C resulted in linear incorporation of ADPR for 30 min, which was totally inhibited by 4 M nicotinamide and 70% inhibited by 5 mM ADPR. PEI-cellulose chromatography of the $^3H\cdot NAD^+$-incubated nuclei after snake venom phosphodiesterase hydrolysis showed half the counts migrating with ADPR and half with AMP, indicating the reaction product is poly(ADPR) with an average chain length of 2 ADPR monomers.

Blastula and pluteus nuclei incubated with $^3H\cdot NAD^+$ were fractionated into nuclear sap (0.15 M NaCl soluble), histone/HMG (0.4 N H_2SO_4 or 0.25 M HCl soluble) and residual proteins. Nuclear sap contained no 20% TCA precipitable material, histone/HMG contained 1.5% (blastula) and 0.35% (pluteus) of the total incorporated ADPR, and the remainder was found in the residual fraction. The specific activity was 0.54 (±0.01) nmoles ADPR/mg protein for blastula histone/HMG, 0.06 nmoles ADPR/mg protein for pluteus histone/HMG, and 35.9 (±2.5) nmoles ADPR/mg protein for residual proteins of both stages.

Supported by the Research Foundation of the State University of New York.

Electron spin resonance studies of protein-methylglyoxal complexes. PETER GASCOYNE, JANE MCLAUGHLIN, AND ALBERT SZENT-GYORGYI.

Pethig, McLaughlin, and Szent-Gyorgyi have shown that the brown color that arises when bovine serum albumin (BSA) is complexed with methylglyoxal (1,2-oxo-propanal) results from the formation of a Schiff base linkage between methylglyoxal and the ϵ-amino groups of the protein lysine residues. Electron spin resonance (ESR) and spectrophotometric measurements were made on the BSA-methylglyoxal complex in order to investigate the link between the brown color and the unpaired spin concentration that also accompanies the treatment of protein with methylglyoxal. The intensities of both the brown color (measured at 318 nm) and the ESR signal (at $g = 2.005$) were directly proportional to the amount of methylglyoxal complexed with BSA. Whereas the intensity of the ESR signal increased rapidly in the presence of molecular oxygen, the optical absorbance remained constant within experimental error. These results show that the ESR signal and the brown color of the BSA-methylglyoxal complex arise from different molecular species. It is proposed that methylglyoxal initially reacts with the ϵ-amino groups of the lysine residues of the protein to form a hemiacetal. The hemiacetal can undergo rearrangement to form either a Schiff base (accounting for the brown color) or else a diol in the ene form. The diol can undergo stepwise oxidation in the presence of oxygen first to form a semidione (accounting for the ESR signal at $g = 2.005$) and then a dione. Such studies are considered to be of direct relevance to the radical signal observed in living systems where methylglyoxal has been observed complexed to proteins. Part of the ESR signal of living systems may arise from complexed methylglyoxal semidione radicals rather than from quinones or flavins as is currently believed.

This work is supported by the National Foundation for Cancer Research.

Partial purification and characterization of a cytotoxic protein from squid (Loligo pealei) *posterior salivary glands.* William R. Kem and James D. Scott.

Homogenization of freeze-dried posterior salivary glands in 1 M ammonium acetate (pH 6) yielded a cytotoxic supernatant fraction that was further purified by molecular sieve chromatography and isoelectric focusing. Cytotoxicity was determined using a 1% suspension of washed rat erythrocytes in 145 mM NaCl, 2 mM $CaCl_2$, and 10 mM Tris, pH 7.4. After incubation of the red cells with toxic samples for 1 hr at 37°C, the suspension was centrifuged, and hemoglobin release measured at 540 nm. All of the hemolytic activity eluted from an AcA-44 Ultrogel column as a sharp peak at 1.8 V_0 (void volume); thus the apparent molecular size of the cytotoxin was about 30,000–50,000 daltons. The cytotoxicity apparently is due to one or more proteins, since it was readily inactivated by boiling or by 30 min exposure to low concentrations of trypsin, pronase, or 1 mM dithiothreitol. The serine protease inhibitor phenylmethylsulfonylfluoride did not affect hemolytic activity. Flat-bed isoelectric focusing demonstrated that the major cytotoxin possessed a pI of 7.2 at 5°C, whereas a minor hemolytic component possessed a pI of 7.8. The hemolytic action of the *Loligo* toxins occurred in the absence of calcium although elevated concentrations (10–40 mM) of calcium progressively prevented hemolysis. Reducing pH to 6 enhanced hemolysis by about 70%, whereas raising the pH to 8.5 had no significant effect. The steady-state hemolysis evoked by *Loligo* toxins was greatly dependent upon temperature, being much greater at 37°C than at 23°, 13°, or 5°C.

This research was supported by NIH grant GM 25849.

Studies of protein synthesis in isolated toadfish hepatocytes. Rita W. Mathews and Audrey E. V. Haschemeyer.

Active hepatocyte preparations have been obtained from toadfish (*Opsanus tau*) by collagenase perfusion based on the method of Seglen. Anesthetized fish maintained with circulating seawater were cannulated through the hepatic portal vein and perfused with 150 ml Hepes-buffered balanced salts (pH 7.8), followed by medium containing 2 mM $MgSO_4$, 1% bovine serum albumin (BSA), and 100 units/ml collagenase Type IV (Sigma). Cells were combed into balanced salts medium and washed by low-speed centrifugation and resuspension. Incorporation of ^{14}C-leucine into protein was assayed in freshly isolated cells in suspension at 0.04 g/ml in a medium containing 0.16 M NaCl, 0.005 M KCl, 0.001 M $MgSO_4$, 0.1% glucose, 25 mM Hepes (pH 7.8), 1% BSA, 0.29 mM each of 19 amino acids, and 0.6 μCi/ml ^{14}C-leucine at 0.014–0.13 mM. At low external leucine concentration, incorporation was linear with time at all temperatures, and reached levels of 30,000 $cpm \cdot hr^{-1} \cdot 100\ \mu l\ sample^{-1}$ at 30°C. The temperature coefficient (Q_{10}) was 5 for the 4°–21°C range and 1.3–1.7 from 21° to 30°C. A similar temperature dependency was obtained with cells in stationary culture 2 days after plating to plastic dishes in serum-free Waymouth's medium. The behavior of the hepatocytes up to 21°C closely resembles that observed for protein synthesis in liver *in vivo*. At high external leucine concentrations, incorporation fell to low levels and Q_{10} increased to 5 (20–30°C) and 20 (15–20°C), suggesting that uptake of the radioactive precursor became rate-limiting under these conditions at low temperatures. Further study of this system will require combined analysis of transport and protein synthetic rates.

Supported by National Science Foundation grant PCM 79-21091.

Histochemical identification of N-acetyl-β-glucosaminidase activity in early embryos of Lytechinus pictus. Kathleen Morgan.

A histochemical technique specific for the localization of N-acetyl-β-glucosaminidase activity was applied to unfertilized eggs and early embryos of *Lytechinus pictus* to determine if the enzyme activity is present in early embryos and if the activity is temporally and/or spatially localized.

N-acetyl-β-glucosaminidase (EC 3.2.1.30) is a lysosomal exoglycosidase specific for non-reducing terminal hexosamines of oligo- and polysaccharides, in invertebrates and vertebrates. The enzymatic activity has recently been found associated with ligatin, a low-molecular-weight, surface-binding protein isolated from vertebrate cell types and also from sea urchin eggs and embryos. Such a lytic activity, if present at the cell surface, could reflect or underlie cellular differentiation.

Lytechinus pictus embryos were fixed briefly in cold 80% ethanol at regular intervals from unfertilized egg through early gastrulation. In the first step embryos were incubated for 2 hr at 37°C in a reaction mixture containing Naphthol-AS-BI-N-acetyl-β-glucosaminide (9×10^{-4} M), or, in the case of controls, the reaction mixture minus the substrate. After washing, the embryos were incubated in a post-coupling reaction mixture containing 1 mg/ml Fast Blue RR salt, a diazo compound which reacts with the released naphthol to produce a blue/violet precipitate at the site of enzymatic activity.

Enzymatic activity is present in the unfertilized egg throughout the cytoplasm, but excluded from the nucleus and not associated with the plasma membrane. From fertilization to early gastrulation the activity remains cytoplasmic and is not selectively segregated to particular cells.

This research was supported by U.S. Public Health Service Grants 2T32GM07231-06 and T32H007098.

The status of mRNA in eggs and early embryos. ANDREW MURRAY AND RONALD SOSNOWSKI.

We measured the translatibility of the mRNA in extracts of *Arbacia punctulata* eggs by adding them to mRNA-dependent reticulocyte lysate (MDL). The eggs or embryos were homogenized in 45.5 mM KCl, 2.3 mM $MgCl_2$, 69.6 mM Kgluconate, 50 mM HEPES, 10 mM EGTA, 618 mM glycerol titrated to pH 6.9 with KOH, and centrifuged at $12{,}000 \times g$ to yield a post-mitochondrial supernatant (PMS). Protein synthesis was assayed as the incorporation of ^{35}S-methionine into TCA insoluble material.

Protein synthesis in this system was linear for 45 min and was 90% inhibitable by edeine, showing that re-initiation occurred on PMS mRNA. By 20 min post-fertilization the PMS-directed incorporation was twice that of unfertilized eggs. A similar increase in polysomal mRNA was detected as increased edeine insensitive incorporation. It remains unclear whether non-polysomal mRNPs contribute to translation in the MDL. To demonstrate masked mRNA we translated extracted RNA and PMS at equivalent concentrations, assuming complete recovery of the RNA. We precipitated RNA by adding one volume of 4 M LiCl, 8 M urea, 0.5 mM EDTA, and 20 mM Tris pH 7.5 at 0°C, and phenol-extracted the pellet. Purified RNA directed 2–4 times more incorporation than the PMS from eggs or early embryos. Since the PMS is non-inhibitory to translation of purified mRNA we conclude that the PMS contains functionally masked mRNA.

The clam *Spisula solidissima* undergoes a change in the pattern of protein synthesis at fertilization. To investigate the protein components of mRNPs Spisula oocyte PMS was passed over oligo-dT cellulose columns to which poly-A, oocyte mRNA, or no added components had been bound. Proteins were eluted with increasing salt concentrations and analyzed on SDS-polyacrylamide gels. The 0.1–2 M eluates from all three columns were identical, while the 4 M NaCl eluate from the oligo-dT-mRNA column showed seven additional stained bands.

Supported by NIH training grant GM 00265.

Inhibition of islet prohormone to hormone conversion by incorporation of arginine and lysine analogs. BRYAN D. NOE, G. ERIC BAUER, MICHAEL N. MORAN, STEPHAN KAHLER, AND JEFFREY B. NEUSTADT.

Previous work using isolated anglerfish (AF) islets has established the existence of biosynthetic precursors for glucagon and somatostatin as well as for insulin. Most polypeptide prohormones have pairs of basic amino acid residues at the prohormone cleavage sites. AF islets were incubated with 3 mM canavanine (CAN), thialysine (TL), and/or norleucine (NL), and ^3H-trp and ^{14}C-ile to determine whether: a) the analogs of arginine (CAN) and lysine (TL, NL) can be incorporated into islet prohormones, and b) prohormone conversion is inhibited by analog incorporation. After incubation of islets, 2 M acetic acid extracts were analyzed for prohormones and hormones by gel filtration. Prohormone-hormone ratios from extracts of tissue incubated with and without analogs were compared. Incubation with CAN alone resulted in 81, 72, and 10% reduction of proinsulin (Pro-I), proglucagon (Pro-G), and prosomatostatin (Pro-SS) conversion, respectively. Incubation with TL alone reduced Pro-I conversion 81%, Pro-G 53%, and Pro-SS 23%. Incubation with NL reduced Pro-I conversion 3%, Pro-G 17%, and Pro-SS 22%. Incubation with CAN + TL reduced conversion of Pro-I 94%, Pro-G 83%, and Pro-SS 46%. CAN + NL reduced Pro-I conversion 77%, Pro-G 73%, and Pro-SS 32%. All results are means of 3 determinations. Arginine, lysine, or their analogs (3 mM), added to lysed secretory granule preparations, did not inhibit conversion of non-analog containing prohormones, indicating that the reduction of prohormone conversion in intact tissue was not due to inhibition of the converting enzyme(s) by the analogs. Moreover, conversion of CAN + TL containing precursors by lysed granules was approximately 90% less than conversion of non-analog containing precursors. Analog-containing prohormones also were significantly less degraded than normal precursors after limited trypsinization. These results indicate that CAN, TL, and NL are incorporated into islet prohormones, resulting in inhibition of prohormone to hormone conversion.

Supported by NIH grants AM-16921, AM-26378, and AM-00142.

Inhibition of L-leucine transport in toadfish liver by various natural amino acids. Roger Persell and Audrey E. V. Haschemeyer.

Liver uptake of amino acids has been studied *in vivo* by monitoring the distribution of ^{14}C-amino acids and of ^{3}H-inulin between liver and blood at short times after pulse injection into the hepatic portal vein. Previous studies have shown that transport of L-leucine at 20°C is a saturable carrier-mediated process operating through a combination of active transport and facilitated exchange. The major competing amino acids are isoleucine and phenylalanine with lesser effects by valine, methionine, and aspartic acid. The present study explores the effect of reduced temperatures on competition characteristics of this system. Toadfish acclimated to ambient summer seawater temperature ($20° \pm 1°C$) were cooled to $10° \pm 1°C$ 1 hr prior to experiments. Under benzocaine anesthesia fish were injected with 2 μCi [^{14}C(U)]-leucine and 4 μCi [^{3}H(G)]inulin in 0.1 ml balanced salts medium, pH 7.8. Leucine concentration was 0.1 mM; competing amino acids were tested at 15 mM. The liver was excised after 5 min, and blood collected from the cut hepatic vein. Ratios of ^{14}C/^{3}H and total recoveries of the two isotopes in liver and plasma were determined. The results showed a high level of inhibition of leucine uptake by isoleucine (63%) and phenylalanine (54%), as at 20°C. Significant increases in inhibitory activity were found with valine (66%), methionine (63%), and aspartic acid (49%), compared with 20°C data. Histidine also was active (36%) at 10°C. The results suggest qualitative changes in the leucine uptake system at reduced temperatures.

Supported by National Science Foundation grant PCM 79-21091.

Influence of water structure on proton diffusion in proteins. Ronald Pethig, Peter Gascoyne, Jane McLaughlin, and Albert Szent-Györgyi.

Electrical conduction and polarization measurements are reported for compressed polycrystalline samples of bovine serum albumin and lysozyme. The dielectric loss peak observed can be interpreted in terms of the existence of hopping charges rather than as relaxation of conventional molecular dipoles. Both the characteristic relaxation time of this dielectric dispersion and the measured steady state resistivity rapidly decrease with increasing protein hydration, and from the direct relationship found to exist between these two electrical parameters it can be concluded that the hopping charges diffuse across the whole bulk of the test samples. The estimated limiting diffusion coefficient for complete protein hydration, and the form of the dielectric dispersion, agree closely with the calculations of Kirkwood and Shumaker (1952) regarding the effects that would arise from the motions of protons on protein surfaces. The involvement of protons, rather than of some other charged species, is also suggested by the electrical conduction studies of other workers. The dielectric dispersion and conductivity can be altered as a result of chemical treatments designed to vary the nature of the protein-bound counter-ions. These studies can also be taken to indicate that protons are involved in controlling the observed electrical properties. Dielectric measurements have also been made to determine the effective dipole moment of the various states of the protein bound water, and it has been found that the observed dielectric relaxation time and steady state conductivity are strongly dependent on the structure of the primary hydration sheath. These studies are directed towards understanding the significant differences in the dielectric properties of normal and cancerous tissues observed in our laboratory.

This work is supported by the National Foundation for Cancer Research.

Changes in the protein synthetic pattern and mRNA population during Spisula *embryogenesis.* TERESE TANSEY AND JOAN RUDERMAN.

The aims of this study were to determine the times when changes in the pattern of protein synthesis occur during *Spisula* development, and to examine whether these changes are controlled at the level of transcription, mRNA processing, or translation. One dimensional SDS gels were used to compare *in vivo* labeled proteins with proteins translated from total, poly A^+ and poly A^- RNA added to reticulocyte lysate. Between first cleavage and 18 hr of development, when the veliger larva has formed, approximately 35 changes in the *in vivo* pattern of protein synthesis were detected. The majority of these occur during the first 6 hr of development as the embryo becomes a swimming gastrula. Most of the *in vivo* alterations in protein synthesis are paralleled qualitatively and quantitatively by changes in the translatable mRNA. Thus mRNA masking plays no obvious role in the regulation of most of the changes in protein synthesis we observe.

Most proteins are encoded by both poly A^+ and poly A^- RNA, although a few messages are predominantly poly A^+ or poly A^- at all the stages examined. Some messages undergo a dramatic switch in adenylation state soon after fertilization. By the time of germinal vesicle breakdown at 10 min after fertilization at least three abundant messages which are poly A^- in oocytes become poly A^+. Earlier work has shown that the translation of these messages *in vivo* increases greatly after fertilization. Experiments are planned to determine whether the altered translatability and the change in adenylation state of these messages are related. By 30 min after fertilization two messages that are poly A^+ in oocytes become poly A^-. Whether these messages encode proteins whose synthesis decreases after fertilization is not clear.

Supported by NSF grant PCM 79 23046 to J. R.

A preliminary characterization of the hemocyanin of the giant sea roach, Bathynomus giganteus. K. E. VAN HOLDE AND M. BRENOWITZ.

Hemolymph was obtained from a specimen of this large isopod by cutting across several legs. The clotted hemolymph was squeezed through cheesecloth and then centrifuged at low speed for 5 min. After dilution 1 : 4 with filtered sea water, analytical sedimentation velocity revealed that at least 95% of the hemolymph protein was a 16S hemocyanin. The hemocyanin was purified by pelleting at 65,000 rpm for 1 hr. Studies of the sedimentation velocity at pH 7.4 (0.1 I Tris, 10 mM Mg^{2+}, 10 mM Ca^{2+}) gave a value of $S^0_{20,w} = 16.7S$. In 0.1 I Tris buffers containing 10 mM EDTA the hemocyanin dissociates progressively with increasing pH. Above pH 9, only a single 4.5S component is observed. That the 16S hemocyanin is the principle form *in vivo* is supported by an experiment in which the hemolymph was centrifuged at 10°C, with an oil overlay to provide a pressure equal to that at a sea depth of 1300 feet, the natural habitat. Only a single component, identifiable as the 16S material, was observed.

We wish to express our thanks to the New York Aquarium for making the specimen available, and to the NIH for Training Grant GM-00265.

NEUROBIOLOGY

Squid axon sodium channels are blocked reversibly by aphantoxin derived from a prokaryotic alga. WILLIAM J. ADELMAN, JR., JURGEN F. FOHLMEISTER, AND JOHN J. SASNER, JR.

Blooms of toxic prokaryotic blue green algae (cyanobacteria) occur from time to time in fresh water lakes in New England. *Aphanizomenon flos-aquae* has been linked to fish kills and fouling during these algal blooms. A toxin, aphantoxin (ATX), has been derived from *A. flos-aquae*, and biochemical and neurophysiological data indicate that ATX is similar to saxitoxin (STX) and tetrodotoxin (TTX) in its chemical properties and mode of action.

In order to determine the site of action of ATX, we applied ATX to voltage clamped axons of the squid *Loligo pealei.* The ATX used in these experiments was derived from algal samples collected from Kezar Lake, North Sutton, New Hampshire, during a bloom essentially unialgal with *A. flos-aquae.* A standard mouse assay commonly employed for saxitoxin in marine bivalves was used as a potency check. On the basis of this assay, artificial seawater solutions were prepared with saxitoxin-equivalent concentrations of 1, 3, 10, 30, and 100 nM. The voltage clamp experiments showed that ATX blocks or reduces the sodium current of the squid axon without effect on the steady-state potassium current. The rise time of the sodium currents (when present) was unaltered by ATX. Recovery of the sodium current amplitude following ATX washout was complete. Using a non-linear least squares curve-fit routine, the fraction of sodium channels blocked as a function of ATX concentration was fitted to a dose-response curve with 1 to 1 stoichiometry having a dissociation constant of 3.47 nM STX-equivalent concentration.

The similarity of ATX action on nerve to that of TTX and STX and its reversibility are important to neurobiologists. The production of a neurotoxin by a prokaryote raises many interesting biological questions.

Are membrane lipids calcium ionophores? A comparison, employing arsenazo III in liposomes, between known ionophores (A23187, ionomycin) and phospholipids, fatty acids, prostanoids, and retinoids. P. ANDERSON, C. SERHAN, E. GOODMAN, P. DUNHAM, AND G. WEISSMANN.

Liposomes that have entrapped the metallochromic dye, arsenazo III, are a sensitive assay system for ionophoresis of divalent cations: the lipid bilayers are impermeable to divalent cations until ionophores are added. By this means we have compared the known calcium ionophores A23187 and ionomycin with membrane phospholipids, fatty acids, prostanoids, and retinoids. Added at micromolar concentrations to preformed multilamellar liposomes (phosphatidyl choline 7: dicetyl phosphate 2: cholesterol 1) both A23187 and ionomycin, as well as phosphatidic acid, linoleic acid, linolenic acid, and two eicosatrienoic acids (precursors of prostanoids) provoked Ca influx, for example, phosphatidic acid: 12 mmoles Ca/(mole lipid$\cdot$min^{-1}). A variety of other phospholipids (*e.g.* phosphatidyl serine), fatty acids (*e.g.* arachidonic acid), prostanoids (*e.g.* PGE_1), retinoids (*e.g.* retinoic acid), and glyceryl ether phosphoryl cholines ("platelet activating factors") were without effect. The membrane lipid ionophores such as phosphatidic acid translocated divalent cations selectively, demonstrating the same rank order as A23187 or ionomycin: Mn > Ca > Sr >> Mg. Membrane lysis did not contribute to the perceived translocation as the liposomes remained impermeable to EDTA, EGTA, arsenazo III, or Mg. Liposomes with phosphatidic acid or the trienoic acids preincorporated at 1–5 mole percent of total lipids also permitted translocation of Ca but not Mg. Release of Ca from liposomes which had entrapped arsenazo III$\cdot$Ca complexes into medium rich in EGTA permitted calculation of efflux induced by ionophores, whether these were added to the outside of liposomes or preincorporated. Data suggest that phosphatidic and trienoic acids (formed in natural membranes after phospholipase activation) act as calcium ionophores in model bilayers, and could play a similar role in cells as "endogenous ionophores."

Dark-adaptation effects on photobehavior of Hermissenda crassicornis *(Gastropoda: nudibranchia).* EDWARD BARNES AND IZJA LEDERHENDLER.

The diurnally-dependent photopositive behavior of the sea snail *Hermissenda crassicornis* may be described in terms of three component phases: initiation of movement, approach to light, and selection of an intensity level within an illumination gradient. We found previously that variations in the photobehavior depend on an individual's past experience with light. We report here the effect of one aspect of this: amount of time in the dark before testing (dark-adaptation). We measured the behavioral components as follows: time to leave a start area (start latency); the time, after starting, to cross within 8 cm from the center of illumination (latency to enter the light); and the time spent in the light within the 8 cm boundary. Animals were maintained on a 12 hr light: 12 hr dark cycle and tested during the light portion of the cycle. Individuals were dark-adapted for 0, 5, or 15 min, and then placed in the center of a shallow aquarium illuminated from above at one end. The resulting gradient decreased at least three orders of magnitude from a maximum of approximately 100 Joules m^{-2} sec^{-1}.

Increased dark-adaptation significantly increased the start latency but did not alter latency to enter the light. After entering the light, however, individuals receiving more dark-adaptation remained in the light for significantly less time. Thus dark-adaptation seems to contribute to reduced photopositivity. This conclusion is also supported by our finding that among animals that entered the light, dark-adapted individuals spent more time in relatively less illuminated areas. While the start latencies were not correlated with the latencies to enter the light, other aspects of the approach behavior, including velocity, turning, and heading, may be important.

The photopositive behavior of *Hermissenda crassicornis* is an important feature of a cellular model of learning that is being developed for this animal. Further detailed study of its mucus trails will help specify the exact nature of the change in behavior.

Supported by IRP, NINCDS, NIH.

Guanosine phosphates and the control of membrane potential in Limulus *ventral photoreceptors.* S. R. Bolsover and J. E. Brown.

Solutions of guanosine phosphates were injected into *Limulus* ventral photoreceptor cells. Where possible, the nucleotides were mixed with the dye Fast Green FCF, which is without effect when injected alone. Analogues of guanosine triphosphate (GTP) had a striking effect on the photoreceptors. Injection of GMP-PNP (guanylyl imidodiphosphate), GMP-PCP (guanylyl (β, γ methylene) diphosphate) and GTPγS (guanosine γ-thio triphosphate) substantially increased the frequency of "quantum bumps" observed in darkness. Injections were monitored visually in a relatively bright light. After this adapting illumination, the amplitudes of both light-induced and drug-induced bumps increased. In some cells, a second bright illumination was necessary before the elevation of dark bump frequency was observed. Sensitivity to light (peak light-induced current per stimulus intensity) was unchanged by GMP-PNP injection. Injections of imidodiphosphate, 5′-guanosine monophosphate and 3′,5′-cyclic guanosine monophosphate were without effect on dark bump frequency. When injected in the light GTP had no effect on the subsequent membrane voltage. However, injection of GTP in the dark produced a slow, smooth depolarization, reaching a maximum about 10 sec after injection and decaying over 1–2 min. The response to a light flash was much attenuated during the GTP-induced depolarization and recovered over a few minutes. Although injection of too large a volume of any solution can cause a depolarization due to membrane damage, we observed the smooth, transient depolarization only after GTP injection. In voltage clamped cells, the steady-state current-voltage relation measured in constant light intersects with that measured in the dark at the reversal voltage ($< +30$ mV). When measured similarly, reversal voltage for the change in membrane current induced by GTP injection ($\sim +55$ mV) was substantially more positive than that of the light-induced current.

Supported by NIH EY-01914 and EY-01915.

Preliminary evidence for taste-aversion learning in the nudibranch mollusc Aeolidia papillosa. M. B. Boyle and L. B. Cohen.

We have undertaken to develop taste-aversion learning in an opistobranch mollusc using a paradigm similar to that used by Sahley, Rudy, and Gelperin on the pulmonate *Limax maximus.* In our experiment, each slug was exposed once per day to two anemones, *Tealia lofotensis* and *Epiactis prolifera*, with 3 hr between presentations. As an aversive stimulus, quinine (5–10 mM) in seawater was administered following 1 min of feeding on one of the two foods. Four of the slugs received quinine paired with one species, the other four with the second species. We predicted the development of a selective aversion to the paired food. Two response measures were used. First, tines before biting into both foods were measured during each trial and the difference of the two tines (paired minus unpaired) calculated. We expected that the mean differences would become significantly positive after training. Second, the slugs were tested in an olfactometer in which the odors of the two anemones were presented simultaneously. A choice score was calculated for each slug based on its position in the box at 10, 15, 20, and 25 min after the start. We asked whether the preferences of the two groups became different. For both measures considered on a day-by-day basis, the data were infrequently significant at the 0.05 level or below (one-tailed *t* test). However, if for each measure the data points from the last six of the nine trials were combined into two groups of three consecutive days, the data was significant at the 0.01–0.04 level. We believe that we have conditioned a selective aversion in these animals, and plan in the near future to work on modifying the paradigm to achieve a more robust effect.

Supported by NIH grant NS0837.

Presynaptic injection of calcium facilitates transmitter release in the squid giant synapse. MILTON P. CHARLTON, ROBERT S. ZUCKER, AND STEPHEN J. SMITH.

Facilitation of transmitter release involves secretion of increasing amounts of transmitter evoked by successive action potentials in a presynaptic terminal. This facilitation could be due to "residual calcium" which lingers in the presynaptic terminal following an action potential. To test this hypothesis we mimicked residual calcium by direct injection of calcium into presynaptic terminals of the squid giant synapse. Current was passed between the barrels of a thick-septum theta-tube microelectrode, one tube filled with 0.5 M $CaCl_2$ and 1.5 M KCl, and the other with 3 M KCl. Electrodes were tested for calcium ejection in a solution of Arsenazo III.

When 5–50 nA was passed through the calcium barrel to inject calcium into the presynaptic terminal the membrane potential of the postsynaptic cell became noisy and was depolarized by as much as 1 mV. This result was routine and indicative of a large increase in spontaneous transmitter release caused by the additional calcium. Synapses placed in low calcium saline gave subthreshold synaptic potentials which sometimes increased 5–10% during calcium injection but were severely depressed for several minutes after the injection.

More consistent results were obtained when transmission was eliminated in 1 mM $CaCl_2$, 7 mM $MnCl_2$ saline and was restored at a restricted area by focal application of calcium from an extracellular pipette located adjacent to the intracellular calcium pipette. In all such experiments intracellular calcium injection caused increased spontaneous release, increased (5–30%) evoked release and long lasting depression of synaptic responses. Twin-pulse facilitation was little affected by the additional intracellular calcium. There was no effect on presynaptic resting or action potentials. Injection of potassium had no effect. The results indicate that additional intracellular calcium can cause facilitation of transmitter release and lend strong support to the residual calcium hypothesis.

M.P.C. and S.J.S. are Steps Toward Independence Follows. Supported by NIH grant NS15114 and a Sloan Foundation Fellowship to R.S.Z.

A GTP binding component regulates discrete wave production in Limulus *ventral photoreceptors: pharmacological evidence.* D. WESLEY CORSON AND ALAN FEIN.

The receptor potential of *Limulus* ventral photoreceptors is composed of discrete waves at low light intensities. Recently we reported that fluoride induced production of discrete waves in the dark. We now report that vanadate ions (VO_3^-) and GTP-γ-S (guanosine-5′-O- (3 thiotriphosphate)) induce the production of discrete waves in the dark and prolong the response to a flash of fixed intensity. The time course of discrete waves induced by vanadate and GTP-γ-S (a hydrolysis resistant analog of GTP) appears to be similar to the time course of spontaneous and light-induced discrete waves. The vanadate and GTP-γ-S induced discrete waves also undergo adaptation in response to a bright adapting light. We tentatively conclude that vanadate and GTP-γ-S induced discrete waves arise from a process similar or perhaps identical to visual excitation.

Microelectrodes with 100 mM solutions of vanadate, GTP-γ-S, or the control substances GTP, ATP, or ATP-γ-S were used for iontophoretic injection. Control substances were without comparable effects on discrete wave production. The effects of vanadate injection were largely reversible within 6 hr. GMP-PNP (guanylyl-imidodiphosphate), a second hydrolysis resistant analog of GTP, did not consistently produce discrete waves in the dark.

GTP-γ-S, vanadate, and fluoride are members of the class of compounds known to activate hormone regulated enzymes through direct activation of GTP-binding regulatory proteins. From the observed effects of these compounds on ventral photoreceptors, we suggest that a GTP-binding regulatory protein is very likely to be involved in the regulation of discreate wave production.

Supported by grants from the NIH and the Rowland Foundation.

An X-ray study of the retinal photoreceptor structure of squid. A. R. Czeto, A. R. Worthington, and C. R. Worthington.

We have investigated the molecular structure of the invertebrate rhabdomeric visual cells of squid by low-angle X-ray diffraction. It is known from previous X-ray studies by Worthington *et al.* (1976, *Nature* **262**, 626) that X-ray patterns can be recorded from squid retina after fixation with glutaraldehyde. This previous study demonstrated that the two-dimensional array of microvilli was hexagonal with $a = 620$ Å and also that the light direction was coincident with the (11) direction of the two-dimensional array. All later attempts to obtain X-ray patterns from live squid retina after transportation from M.B.L. to our X-ray laboratory at Pittsburgh have failed. In the present study the X-ray experiments were run at M.B.L. The squids were first dark adapted for 45 min. In the X-ray experiments a 1 mm strip was cut from the freshly dissected eye and mounted in a specimen chamber with excess Ringer's solution. The X-ray exposure was started about 1.5 hr after the arrival of the squid in the M.B.L. holding tank. Slit collimation with exposure times of 1 or 2 hr was used to explore whether low-angle X-ray diffraction could be recorded from the intact, untreated strips of squid retina. Initially, only poor patterns were obtained, indicating that the microvilli array was disordered. It was soon found that the ordering of the array was extremely sensitive to the chemical composition and the osmolarity of the Ringer's solution. Poor X-ray patterns were routinely obtained using the various artificial sea waters even after adjusting the osmolarity of the solution to the osmolarity of the vitreous humor of the squid eye, namely about 900 milliosmoles. A Ringer's solution based upon a study by Robertson (1953, *J. Exp. Biol.* **30**, 277) was designed to match the osmolarity of the squid eye. We report here that excellent X-ray patterns showing diffraction orders 2–8 of $d \approx 640$ Å were obtained from intact untreated squid retina when using this Ringer's solution. The diffraction extends out to a minimum spacing of about 35 Å, which is typical of membrane diffraction.

This work was supported by NIH grant NS 09329.

Field experiments on electrically evoked feeding responses in the dogfish shark, Mustelus canis. Benjamin G. Dawson, Gail W. Heyer, René E. Eppi, and Ad. J. Kalmijn.

From previous laboratory experiments, we learned that sharks, skates, and rays have an electric sense that enables them to detect voltage gradients as low as 0.01 μV/cm within the frequency range from DC up to 8 Hz. The animals use their electric sense in predation, cuing in on the bioelectric fields commonly produced by fish and aquatic invertebrates. To quantify the response, we analyzed the feeding behavior of the shark *Mustelus canis* in Vineyard Sound off Cape Cod, Mass. An electrode panel was embedded in the ocean substrate in a water depth of 2–3 m. Two salt-bridge electrodes, simulating a small prey fish, were placed 2 cm apart at a distance of 15 cm from a centrally located odor source. Another pair of salt-bridge electrodes, simulating a larger fish, were placed 5 cm apart at a distance of 30 cm on the other side of the odor source. DC current of 8 μA was applied to either one or both pairs of electrodes. Observations were made at night from a Boston Whaler with a glass-bottomed observation well. Liquified herring chum attracted and motivated sharks. Markings on the electrode panel enabled observers to measure the distances from the electrodes at which the sharks initiated attacks. Data were recorded on the size of the sharks (30–40 cm pups or 90–120 cm adults), the vigor of response, and the pattern of attack. Out of 136 responses, first-year sharks attacked the 2-cm electrodes 49 times from a distance of 15 cm or more measured along the dipole axis, which corresponds to a sensitivity of 0.04 μV/cm or higher. Of these 49 responses, 16 were from at least 18 cm, yielding a sensitivity of 0.02 μV/cm or better. Out of 112 responses, larger sharks attacked the 5-cm electrodes 44 times from a distance of 30 cm or more, equivalent to 0.01 μV/cm or better. Of these 44 responses, 15 occurred from at least 38 cm measured along the dipole axis, establishing a sensitivity of 0.005 μV/cm or better. With both pairs of electrodes on, the small sharks, after attacking the 2-cm electrodes, often veered away from the 5-cm ones. In sum, the results support the conclusion that these sharks, once motivated by odor, rely heavily upon their keen electric sense in executing their final strikes.

(This work was conducted as part of Kalmijn's project on electric and magnetic detection under contract with the Office of Naval Research, N00014-79-C-0071.)

Positional correlation of synaptic boutons in pairs of mechanosensory cells in the leech. Susan A. DeRiemer and Eduardo R. Macagno.

In the leech, each segmental ganglion contains six mechanosensory cells which respond to light touch (T-cells). They make synaptic connections with one another and onto identified motor neurons, among others. Our work addresses the question of whether the synaptic contacts between T-cells are localized or distributed throughout their branching fields. Each of two ipsilateral T-cells in individual segmental ganglia were injected with one of two dyes: horseradish peroxidase (HRP) and Lucifer Yellow. The HRP reaction was carried out in saline prior to fixation. Afterwards the ganglia were mounted in methyl salicylate and viewed under compound optics. The HRP cell was first drawn on a computer-coupled microscope. The positions of synaptic boutons of the Lucifer-filled cell which came into close proximity with branches of the HRP cell were then determined using a 100X oil immersion objective and marked on the computer-drawn cell. Our results indicate that there are numerous close appositions between pairs of T-cells. Analysis of four such T-cell pairs shows that about 100, or 40% of the total synaptic boutons of any one T-cell, are located close to the branches of the other T-cell. These appositions were distributed in all cases throughout the entire T-cell field, with no apparent localization to a definable subset of branches or to any part of the branching field of a cell.

This work was carried out under the auspices of the Neural Systems and Behavior Post-course Research Program.

The central projections of the lateral eyes of Balanus nubilis *(Pacific giant barnacle) differ from those of the median eye.* Kathleen A. French and Ann E. Stuart.

Three simple eyes, one median and two lateral, comprise the visual system of the Pacific giant barnacle. This system is organized to respond to the dimming of light, and the second- and third-order cells of the median pathway, located in the supraesophageal ganglion (SEG), have previously been identified. The projection of the lateral eye photoreceptors (LPRs) to cells in the SEG was studied, and the integration of information from the lateral and median eyes compared.

Cobalt filling showed that LPRs end entirely ipsilaterally, unlike the median (M) PRs, which bifurcate and terminate in both hemiganglia. The terminal arborizations of the LPRs and MPRs overlap in the ganglion. Two types of second-order cells ("inverting" or I-cells) are distinguished by their input from the three eyes. One type (the "symmetrical" or I_s-cell) depolarizes in response to shadowing any of the eyes, while the other type (the "asymmetrical" or I_{as}-cell) depolarizes when the median or ipsilateral, but not the contralateral, eyes are shadowed. The median eye provides the strongest input to the I_s-cell, while the ipsilateral eye is dominant for the I_{as}-cell.

Certain third-order cells ("amplifying" or A-cells) of the median visual pathway project out of the SEG via the circumesophageal connectives; dimming produces a burst of impulses in these cells. Shadowing each of the three eyes produces a synaptic potential and impulses in an A-cell, although the input from the median eye is dominant. Other unidentified cells in the vicinity of the I- and A-cells respond differently to shadowing the three eyes. Integrating input from three eyes may provide the barnacle with spatial information that one eye could not detect.

Supported by NIH grant EY03347 to A.E.S.

Interaction of external transition metal ions and the mobile gating charges of Na and K channels in squid axon. Wm. F. Gilly and Clay M. Armstrong.

Voltage clamp experiments on perfused axons show that 30 mM $ZnCl_2$: 1) slows ON kinetics of Na channels as they open, 2) does not alter OFF kinetics as they close, and 3) slightly reduces the maximum attainable conductance. Gating charge movement (I_g) is affected in a parallel manner: 1) I_g ON is slowed, 2) I_g OFF is unaltered, and 3) maximum charge movement is unchanged. K channels are affected in the same way but are more sensitive to Zn. Cd(II) slows ON kinetics of both channels about as effectively as does Zn. Hg(II) also acts like Zn, but at μM concentrations and irreversibly. 5 mM Cu(II), Mn, Ni, and Co do not affect K channels, nor does 12 *vs.* 50 mM Ca.

We postulate that the divalent IIb transition metal ions (Zn, Cd, Hg) can electrostatically interact with mobile gating charges of Na and K channels. Gating charges are of negative sign and at resting voltages are exposed at the membrane's outer surface. Depolarization leads to inward migration of these negative charges, generating the outward I_g ON transient. External Zn ions stabilize the closed channel conformation by electrostatically interacting with the gating charges in the resting position. The attraction can be overcome by sufficient depolarization; I_g ON is thus slowed but not prevented. Once gating charges migrate inward, the Zn ion is no longer electrically visible to them; I_g OFF and channel closing rate are therefore almost unaltered. The electrically close approach of only IIb metal ions to the resting gating charges may be due to specific covalent binding of these very polarizable and reactive ions by the channel proteins.

The binding sites need not carry a fixed charge. Knowledge of zinc-binding enzymes suggests neutral imidazole nitrogens of histidine residues as likely candidates.

Phospholipid synthesis in axoplasm from the squid giant fiber. Robert M. Gould.

Larrabee and Brinley (1968, *J. Neurochem.* **15**: 533–545) have demonstrated that axoplasm extruded from the squid giant axon catalyzes the formation of several phospholipids, *i.e.*, phosphatidylinositol, phosphatidylethanolamine, and phosphatidic acid from ^{32}P-inorganic phosphate precursor. I have used several other radioactive precursors to further define axoplasmic phospholipid biosynthetic capabilities. With 2-^{3}H myoinositol, ^{14}C-serine, and ^{14}C-cytidine triphosphate (CTP) as precursors, formation of phosphatidylinositol, phosphatidylserine, and cytidine diphosphate diglyceride (CDP-diG) was demonstrated. When unlabeled CDP-dicaproin was included in the incubation (with [2-^{3}H]myoinositol) three to five times more labeled lipid was formed during the 2 hr incubation. Similarly, phosphatidic acid derived from egg lecithin markedly stimulated lipid formation (a 3- to 7-fold increase) when either CTP or serine was the labeled precursor.

Additional experiments further characterized the enzymes of lipid metabolism in axoplasm. Inositol transferase activity was measured at 1 mM myoinositol concentration, and the synthesis of phosphatidylinositol was linearly dependent on time and on protein concentration. Exogenous CDP-diG was required for this synthesis, and $MgCl_2$, sodium deoxycholate, and potassium chloride increased the rate of the reaction. With 5 mM γ-^{32}P-ATP as precursor, diglyceride kinase, and phosphatidylinositol kinase activities were demonstrated in axoplasm. The activities of these enzymes could only be demonstrated when exogenous lipid substrates were added.

These results demonstrate that many acidic phospholipids can be synthesized in the axon. This is in contrast to proteins which are not synthesized in the axon.

Better fluorescent probes for optical measurement of changes in membrane potential.

A. Grinvald, R. Hildesheim, R. Gupta, and L. B. Cohen.

We have attempted to improve the sensitivity of voltage-sensitive probes by design, synthesis, and testing of 50 new dyes. For transmission measurements a new oxonol dye, bis-[1-p-sulfophenyl-3-methyl-5-pyrazolone-(4)]-p-phenyl pentamethine oxonol (RH-155), may prove useful. This dye is somewhat less sensitive than the best merocyanine-rhodanine dyes but because of its different structure (three negative charges) it may be appropriate when merocyanines are inapplicable. New styryl dyes are the best fluorescent dyes tested thus far on squid axons. One of these is (anhydro-4-(4′-p-dibutylaminophenyl-buta-1′:3′ dienyl) 1-δ-sulfobutylpiridinium hydroxide) (RH 160). RH 162 is the γ-sulfopropyl analogue of RH 160. RH 160 is sparingly soluble in water but 4×10^{-6} M gave large fluorescence signals ($S/N = 8$–10). Its bleach time was 2000 sec and its kill time 1000 sec. RH 160 is slightly more soluble and has a very large fractional change. Preliminary spectroscopical experiments showed a biphasic action spectrum for both transmission and fluorescence signals. The cross-over points were different for the two signals. In addition, when the excitation wavelength was the same, signals from the blue portion of the emission wavelengths were of opposite direction relative to those obtained at the red tail of the emission. These results are compatible with at least two mechanisms, electrochromism in both the absorption and fluorescence (Loew *et al.* 1979, *Nature* **281**: 497–499) or dye movement in the membrane in the direction of the electric field (normal to the membrane). Additional experiments are required to establish the correct mechanism.

Supported in part by NIH grant NS08437 and NS274-79 and a grant from the U.S.-Israel Binational Science Foundation.

Calmodulin from the axoplasm of the squid. James F. Head and Benjamin Kaminer.

We have purified calmodulin from the axoplasm of the giant axon of the squid *Loligo pealei* using affinity chromatography on rabbit skeletal muscle troponin-I coupled to Sepharose 4B. Calmodulin is selectively bound to the troponin-I in the presence of calcium and is eluted by inclusion of EGTA in the buffer. Our identification of the protein as calmodulin is based on its common mobility with pure bovine brain calmodulin on SDS and alkaline urea gel electrophoresis, its ability to activate bovine brain calmodulin dependent phosphodiesterase and its amino acid composition including a single trimethyl lysine residue per molecule. The protein also binds four calcium ions per molecule, as do the calmodulins isolated from other tissues. Densitometric gel scanning of homogenates of whole axons show calmodulin represents 0.25% of the total protein of the axon.

This work was supported by NSF grant PCM 7904600.

An X-ray study of fish nerves. H. Inouye, A. R. Worthington, and C. R. Worthington.

We have studied the nerve myelin structure of three marine fishes by low angle X-ray diffraction. Previous X-ray studies generally refer to the fresh water fishes. The X-ray patterns of central nervous system myelins from vertebrates show the first five orders of diffraction of $d \approx 150$ to 160 Å with the intensity variation of $I(2) > I(4) >> I(3)$, where $I(h)$ refers to the integrated intensity of the diffraction order h. The X-ray patterns of nerve myelin from fresh water fish are however anomalous in that $I(2) > I(4) \approx I(3)$, that is, there is a strong third order reflection. In the present study, X-ray patterns were recorded from marine teleosts, toad fish and sculpin, and from the elastmobranch skate. The Ringer's solutions were a calcium-free marine teleost solution and a skate Ringer's solution which contained 333 mM urea. The optic nerve and a branch nerve (a fin nerve) of toad fish and the optic nerve and spinal cord nerve of sculpin were examined. These marine teleost nerves had a repeat distance $d = 153$–155 Å and had the same intensity variation as shown by fresh water fish nerves. The X-ray patterns of the two marine teleosts did not change over 7 days. The optic nerve and a branch nerve (a lateral line nerve) of skate were examined. The skate nerves had larger repeat periods: $d = 167$ Å for optic nerve and for spinal cord and $d = 177$ Å for the branch nerve. Moreover, the skate nerves had a different intensity variation from the other fish nerves: the intensity variation was identical to that of the central nervous system myelins from the other vertebrates, namely, $I(2) > I(4) >> I(3)$.

In an earlier X-ray study on fish nerves, Blaurock and Worthington (1969, *Biochem. Biophys. Acta* **173**, 419) identified a second phase with a larger spacing of $d \approx 180$ Å which was superimposed on the original pattern. The toad fish and sculpin nerves did not show this second phase, but the skate nerves readily show this pattern. In the skate the original pattern at first dominant but the larger period pattern with $d \approx 200$ Å replaces the original pattern within 1 day.

This work was supported by NIH grant NS 09329.

An ion-permeable channel produced by venom of the fanged bloodworm Glycera dibranchia. Bruce L. Kagan, Harvey B. Pollard, and Robert B. Hanna.

Venom from the poison glands of the polychaete annelid *Glycera convoluta* has been shown to dramatically increase the frequency of miniature endplate potentials at the frog and crayfish neuromuscular junctions without causing detectable ultrastructural changes. We report that addition to *Glycera dibranchia* venom to one side of a lipid bilayer results in formation of ion-permeable channels in the membrane. *Glycera dibranchia* from Maine were immobilized on ice and the poison glands dissected free. Glands were then homogenized in 10 mM sodium phosphate buffer, pH 7.2, and centrifuged at $48{,}000 \times g$ for 30 min. The supernatant was passed over Sephadex G-25, and 1–10 μl of the void volume fraction ($MW > 5000$) was added to one side of a planar phospholipid bilayer membrane. The final concentration of venom was about 5 milliglands/ml. The conductance of a single channel is about 330 pmho in 0.1 M KCl and is ohmic. The channels exhibit moderate (but not ideal) cation selectivity in NaCl or KCl gradients. Other selectivity measurements suggest that Ca^{2+} and Mg^{2+} are also permeable. The channels show a slight voltage sensitivity. The steady state conductance at −60 mV (side opposite venom) is about 6 times the conductance at +60 mV. We suggest that these channels in the venom may evoke transmitter release at neuromuscular junctions either by (1) depolarizing the pre-synaptic terminal and thus opening voltage-dependent Ca^{2+} channels, or (2) directly allowing Ca^{2+} to enter the terminal. The Ca^{2+} requirement for the venom's effect at neuromuscular junctions is consistent with either mechanism. Black widow spider (*Latrodectus*) venom is known to produce similar effects on neuromuscular junctions and lipid bilayers. Although worms bear little apparent resemblance to spiders, the single channel conductances and ionic selectivities of the channels found in the venoms of *Glycera* and *Latrodectus* are strikingly similar.

(Supported by NIH grant 5T32 GM 7288 to B.L.K. and SUNY Research Foundation award 7142 to R.B.H.)

Coupling between horizontal cells in the carp retina examined by diffusion of Lucifer yellow. AKIMICHI KANEKO AND ANN E. STUART.

The carp retina has four morphological types of horizontal cells (HCs): H1, H2, and H3, all connecting to cones; and a rod HC. Because each type connects with a different set of photoreceptors, it can be identified from its spectral responses. HCs have large receptive fields thought to be due to their electrical coupling. To test this hypothesis, and also to determine whether the coupling occurs only among cells of the same type, we examined diffusion of Lucifer yellow, a fluorescent marker dye, after it had been injected into each type of HC. The penetrated cell was identified by its responses to narrow-band monochromatic lights from green (565 nm), yellow (585 nm), and red (635 nm) light-emitting diodes. As previously reported, H1 cells gave luminosity (L-) type responses with highest sensitivity to red light, rod HCs gave L-type responses with highest sensitivity to blue-green, H2 cells gave biphasic chromaticity (C-) type responses, and H3 cells gave triphasic C-type responses.

In 39 of 56 injections into the cell body, Lucifer yellow spread to immediately surrounding cells and, in some cases, even to secondary neighbors. In all preparations the diffusion was limited to the cells of the same morphological type as the injected one. When dye was injected into the expanded axon endings of the HCs, it diffused to certain other axon expansions, so that the appearance in flat-mounted retinas was of a coarse meshwork. This result indicates that neighboring axonal endings are also coupled and that the coupling is limited, probably to the same cell type. Axons with expanded endings were seen in almost all rod HCs, a finding not previously reported.

Supported by a Rand Fellowship to A.K. and by NIH grant EY03347 to A.E.S.

Recording from the Limulus *ventral eye* in situ: *is there a circadian rhythm?* EHUD KAPLAN, RANJAN BATRA, AND ROBERT B. BARLOW, JR.

The sensitivity of the lateral eyes and median ocelli of the horseshoe crab, *Limulus polyphemus*, increases at night and decreases during the day. The brain imposes this circadian rhythm on the lateral eye via efferent optic nerve fibers which affect both the physiology and the morphology of the photoreceptors (*Science* **197**, 86–89, 1977). In the ventral eye nerve, small fibers terminate on the photoreceptors in what are believed to be neurosecretory synapses, which presumably mediate efferent activity.

We investigated the possibility that the ventral eye undergoes circadian changes in sensitivity. We recorded responses to brief flashes from the ventral eye "wart" *in situ* under constant darkness during the day and at night. No surgery or anesthesia was used. Thus far we have failed to detect any cyclic changes in visual sensitivity. In other experiments we excised the ventral eye and recorded light responses from the unsheathed nerve, using a suction electrode. Shocking the proximal stump of the nerve to activate the efferent fibers did not affect the recorded responses. Likewise, intracellular responses from impaled photoreceptors in the excised ventral eye were unaffected by electrical shocks to the nerve, except for a 10–20 mV depolarization that subsided in about 10 min. Finally, we attempted to influence the light responses of the ventral eye by using octopamine, which B. Battelle (National Eye Institute, N.I.H.) recently found in the efferent fibers of the ventral eye. Although we found octopamine to increase the ERG from the lateral eye, neither the intracellular nor the extracellular responses from the ventral eye were affected by a wide range of octopamine concentrations.

We conclude that under the conditions of our experiments the neurosecretory fibers of the ventral eye nerve do not affect the visual sensitivity of the ventral photoreceptors.

Supported by NIH grants EY188, EY108, EY667, and NSF grant BNS77-19436.

Octopamine increases the ERG of the Limulus *lateral eye.* LEONARD KASS AND ROBERT B. BARLOW, JR.

When the horseshoe crab is kept in constant darkness, the lateral eye produces larger electroretinographic responses (ERG) during the night than during the day (Barlow *et al.*, 1977, *Science* **197**: 86–89). The elevated retinal sensitivity at night is mediated by efferent activity in the optic nerve trunk. During the day, the efferent activity stops and the sensitivity returns to a low, constant level. Pulses of current delivered to the distal end of the cut optic nerve during the day simulate the efferent activity and elevate the ERG to the nighttime level.

B. Battelle (National Eye Institute, NIH) found high endogenous levels and the synthesis of octopamine in the lateral and ventral eyes of *Limulus.* She also found that in the ventral eye octopamine is localized within what appear to be small-diameter efferent fibers and terminals.

We report here that injecting octopamine during the day beneath the cornea of the lateral eye *in situ* increases the amplitude of the ERG. Octopamine (1–10 μM) injected at 1 μl/min for 15 min increased the amplitude of the ERG 2–4× the daytime level, equivalent to about half the elevated nighttime level. Clozapine (25 μM), a demonstrated antagonist of octopamine, reversibly decreased the ERG amplitude of the lateral eye at night. During the day, clozapine (25 μM) blocked the increase in the ERG by octopamine (1 μM). Elevation of the ERG by octopamine injection is reversible, is graded with concentration, and exhibits a time course similar to that caused by the endogenous efferent activity.

Supported by the Grass Foundation, NIH grant EY 00667, and NSF grant BNS 77-19436.

Are there membrane surface charges in the vicinity of the sodium pump? GEORGE R. KRACKE AND PAUL DE WEER.

According to the diffuse double-layer theory of Gouy and Chapman, the magnitude of the surface potential resulting from fixed charges on the exterior of the cell membrane will vary with the ionic composition of the bathing solution, becoming smaller as ionic strength is increased. Consequently, the attraction toward, or repulsion from, the membrane that ions undergo as a result of this surface potential will also decrease as ionic strength is increased. We have exploited this phenomenon in an attempt to determine whether the membrane of the squid giant axon bears external net charges in the vicinity of the sodium pump. The rate of inhibition of the sodium pump by cardiotonic steroids, which follows pseudo-first-order kinetics, was taken as a measure of the effective concentration of these compounds at the membrane/solution interface. Three artificial seawater solutions of normal, low, and high ionic strength were prepared. In the normal ionic strength (NIS) solution, 90% of the usual NaCl (425 mM) and $MgCl_2$ (50 mM) was replaced with N-methylglucamine-HCl. In the low ionic strength (LIS) solution these ions were replaced with sucrose, and in the high ionic strength (HIS) solution with $MgSO_4$. The three cardiotonic steroids used were uncharged ouabian, anionic actodigin hemisuccinate (CS^-), and cationic digitoxigenin aminoglycoside (CS^+). We found the rate of pump inhibition by ouabain, relative to that at NIS, to be 0.67 at LIS and 2.8 at HIS. This variation must be ascribed to chemical effects of the bathing solution changes. However, the corresponding relative rates for CS^- were 0.42 at LIS and 6.6 at HIS, suggesting that the relative concentration of anions at the interface was reduced to 0.63 in LIS seawater and enhanced 2.4-fold in HIS. This is to be expected if the membrane in the vicinity of the sodium pump carries negative charges at a density of one electronic charge per 160 to 480 (average 280) Å^2. Preliminary data with CS^+ suggest that, at least at HIS, a pattern opposite to that described for CS^- is followed, as expected. Further studies with a variety of charged steroids are in progress.

Supported by NIH and the Grass Foundation.

Inside-out voltage clamp in the squid giant axon. J. LÓPEZ-BARNEO, R. D. MATTESON, AND C. M. ARMSTRONG.

In the study of membrane channels by noise analysis, reduction of background noise is essential. We have developed a new voltage clamp that is potentially quieter than the conventional one and is well suited to noise measurements, which require isolation of a small patch of membrane. This clamp has been tested by recording Na and K voltage clamp currents.

Segments of axons were internally perfused and three electrodes were inserted: 1) a pipette to record the internal voltage (V_i) 2) a platinized current wire and 3) a glass pipette to record currents through patches of membrane. This pipette was bent at 90° in the last 150–200 μm, and had a tip diameter of 20–30 μm. The V_i pipette was wired to a clamp amplifier which maintained the axon interior at virtual ground potential. The external voltage (V_o) was measured by an external electrode, and the membrane voltage (V_o-V_i) was then clamped by means of a second clamp amplifier (CA), whose output was wired to large external current electrodes by way of a 200 ohm resistor. Total membrane current (I_t) was measured as the IR drop across the resistor. Current from a small membrane patch (I_p) was gathered by the bent pipette, and fed to a high gain current-to-voltage converter.

The major advantages of this design are: a) stray capacitance of the V_i pipette is essentially eliminated, thus improving the frequency response; b) a low resistance path between the external current recording electrode and ground, present in the conventional clamp, is eliminated, thereby reducing noise; c) potential inside the axon is the same as in the bent pipette, eliminating stray capacitance effects in this electrode.

Na and K currents recorded as I_t were of similar magnitude and time characteristics to the ones recorded with conventional technique. Na currents recorded through the patch are quite similar to I_t. Thus far we have not achieved good isolation of a membrane patch, but perfusion with low conductivity solutions improves isolation and increases I_p (up to 35 nA). Seventy successive I_p records at the same potential were taken to calculate the ensemble variance of current fluctuations. Our analysis suggests that an important source of the fluctuations recorded thus far has been a small variation in the holding potential. Such sources of error must be carefully eliminated before making inferences about the contribution of the stochastic opening of the Na channels to the current fluctuations.

Temperature effects on peak and steady state sodium currents in squid giant axons. RICK MATTESON AND CLAY M. ARMSTRONG.

We have studied the effects of temperature on Na currents and Na-channel gating currents with the intention of estimating the energy barrier encountered either by a mobile charged gating particle or an Na ion as it traverses the membrane field. Some preliminary results indicate that Q_{10} measurements of many Na-channel properties are difficult to interpret since these measurements do not reflect the activity of a single process.

A decrease in temperature in the range of 15°–1°C decreases the peak Na current generated by voltage clamp steps to +20 mV. The Q_{10} of this effect range between 1.83 and 2.44. Temperature changes had little or no effect on the steady state sodium current at +20 mV in inactivation intact axons. In the absence of inactivation (*i.e.* after pronase treatment) steady state Na currents had a large Q_{10} (about 2.0).

Peak Na currents could be increased by delivering large positive prepulses (to + 100 mV) 20–30 msec before the test pulse. Steady state Na currents were essentially unchanged by this procedure. This potentiation effect was more pronounced at low temperatures: at 1°C peak current could be increased by up to 40% whereas at 12°C the increase was about 10%. In the absence of a prepulse, we often observed Na currents that had both a fast and slow activation phase, indicating that some channels may turn on very slowly. Following a prepulse the slow activation phase is eliminated.

One consistent explanation of these results involves the hypothesis that there is a second type of Na channel that normally activates very slowly and does not inactivate. The activation kinetics of these putative slow channels are presumably increased by positive prepulses so that they may contribute to peak Na current. Decreases in temperature seem to transform normal channels into slow channels, accounting for some of the decrease in peak Na current. As a result of this phenomenon, Q_{10} measurements of peak sodium currents are difficult to interpret, since they do not reflect a single process such as temperature effects on single-channel conductance.

Oscillation-free compensation for the series resistance in voltage clamped squid axons. JOHN MOORE, DAVID TAUCK, AND MICHAEL HINES.

In their classic voltage clamp experiments on squid axons, Hodgkin, Huxley, and Katz observed a small resistance, R_s, in series with the membrane and showed that it causes the voltage across the membrane to deviate from the desired command potential by an amount equal to the product of R_s and the membrane current density, I_m. By including a signal proportional to I_m in the control circuit, in principle they could compensate for the unwanted voltage drop across R_s. In practice full compensation was rarely used because of the danger of destroying the axon by oscillations of the voltage clamp. Most investigators still employ this method of R_s compensation and use only partial ($\sim \frac{2}{3}$) compensation.

For accurate measurement of the kinetics and amplitude of membrane conductances, full compensation is necessary. We developed a method to allow complete compensation during ionic current flow without oscillations or ringing. An electronic bridge circuit subtracts from I_m a current equal to the capacitive current, I_c; we apply a fraction of the bridge output, the ionic component of I_m, to the summing point of the control amplifier through a potentiometer set to a value proportional to the measured value of R_s. Such a circuit is so stable that a 2- to 3-fold over-compensation for R_s is obtainable without ringing. The only compromise is that the R_sI_c error from the capacitive component of I_m still causes a lag of a few μsec in the membrane potential rise following a command step.

With this method, we obtain very accurate records of ionic membrane currents. Compared to the uncorrected records, the sodium current is markedly decreased and slowed in the negative conductance region; at all other potentials the currents are increased in magnitude.

Electrical membrane properties of single and two-cell preparations from Chironomus *salivary gland.* ANA LIA OBAID AND BIRGIT ROSE.

Single and two-cell preparations were obtained from salivary glands of *Chironomus* larvae. These preparations' long-term stability in terms of electrical parameters such as resting potential (−30 to −50 mV) and input resistance (4–8 MΩ) enabled us to determine nonjunctional (r_m) and junctional (r_j) membrane resistances under various experimental conditions, in particular those known to affect cell-to-cell coupling. In cell pairs with coupling coefficients close to 1.0, r_j ranges between 50 and 400 kΩ (129 ± 23 S.E., $n = 23$), with r_m usually $20\text{–}30 \times r_j$. These values are of the order predicted by cable analysis of measurements on the intact gland. In the intact gland, exposure to Propionate medium (pH 6.5) lowers intracellular pH (pH_i)—as measured with pH-glass microelectrodes—and uncouples cells. In the isolated cell pairs, r_j under these conditions increases by at least 3 orders of magnitude, while r_m decreases several fold. In the organ, increasing pH of the Propionate medium to 7.4 raises pH_i from 6.5 to 7.3 and recouples cells, at times only transiently. Similarly, in the isolated cell pairs r_j transiently decreases from 100 MΩ to 400 kΩ, with a subsequent renewed rise up to 11 MΩ. pH_i during these times of renewed uncoupling or renewed increase of r_j is found to be unaltered at 7.3, indicating that pH_i does not control junctional resistance in this case. Treatments which raise intracellular Ca^{2+}, such as NaCN or alkalinization by washout of Propionate medium, uncouple the cells in the gland and raise the value of r_j in the cell pairs up to 50 MΩ.

The cell pairs obtained from *Chironomus* salivary gland are a viable preparation, well suited for electrophysiological studies. The data obtained so far from cell pairs are in concordance with and confirm the results obtained with the organ.

Mechanics and energetics of contraction in striated muscle of the sea scallop, Placopecton magellanicus. JACK A. RALL.

The striated adductor muscle is responsible for the characteristic propulsive swimming behavior of scallops. This muscle is also interesting because contractile force is thought to be under Ca^{2+} control via thick filaments, in contrast to the exclusive thin-filament regulation of vertebrate striated muscle.

The purpose of the present study was a general mechanical and energetic characterization of this muscle. Animals were kept at 12°C in continuously running sea water. Experiments were conducted at 10°C in aerated sea water on isolated muscle bundles ($n = 7$) with average dimensions of $3.1 \times 0.25 \times 0.11$ cm weighing 87 ± 10 mg, from scallops with an average shell length of 9.3 cm (about 4 years of age). Energy utilization was derived from measurements of the change in muscle temperature during contraction and relaxation, excluding recovery, using thermopiles. Maximum isometric tetanus force averaged 132 ± 5 mN/mm^2 with a twitch to tetanus ratio of 0.58 ± 0.02. In a twitch, time to peak force development was 87 ± 2 ms and time to half relax from peak force was 104 ± 2 ms. Muscles stimulated repetitively for 1.5 sec displayed a pronounced fatigue, *i.e.*, force at last stimulus was $51 \pm 5\%$ of peak tetanus force. A twitch elicited after a tetanus was transiently potentiated with peak force ($132 \pm 8\%$ of control twitch) occurring 5–15 sec post-tetanus and returning to control values by 3 min post-tetanus. Twitch energy liberation averaged 7.6 ± 0.5 mJ/g with a force to heat ratio of 10.6 ± 1.0 (dimensionless). This ratio decreased by 25 ± 2% when measured in twitches 3 min post-tetanus. Thus the same twitch force after a tetanus utilized 25% more energy than before a tetanus. In general the striated adductor muscle displayed properties consistent with its function of brief, rapid force development rather than slow, maintained force generation.

This research was supported by the Ohio State University College of Medicine.

Orthogonal polarization sensitivities of squid photoreceptors: implications for a retinal design. WILLIAM M. SAIDEL.

Single- and multi-unit responses were recorded with suction electrodes from squid photoreceptor axons in an isolated eye preparation. Individual units responded to graded flashes and light steps with graded trains of spikes. The number of spikes and duration of the train depended upon state of retinal adaptation and relative angle of plane-polarized light. For example, a dark-adapted photoreceptor generated an 11.0 sec train of 186 spikes to a flash. When light-adapted, the response to a similar stimulus was a 0.5 sec train of 25 spikes. Responses to steps of light displayed a changing character with adaptation. Dark-adapted photoreceptors responded to a series of graded steps with a higher frequency of spikes. As the cell adapted to light, the response was transformed into a phasic *on/off* response. With bright background illumination, only a phasic *on* response was elicited by a step. Individual photoreceptors also responded as analyzers of polarization. Spike trains from the same receptor in response to orthogonally plane-polarized light steps varied in frequency from 25–40%. Maximal variation in frequencies occurred with orthogonally polarized stimuli. As predicted from the orientations of microvilli in photoreceptor outer segments, two classes of receptors were observed whose maximal sensitivities in the polarization domain varied by 90°. Polarization sensitivity of a single photoreceptor may be considered as a broad band filter. Thus, the squid retina contains a two-channel mechanism for analyzing objects in a watery world illuminated by naturally polarized submarine light. Preferential sensitivity of photoreceptors to polarized light along angles parallel and perpendicular to the water surface would minimize stimulation by scattered or reflected light having random planes of polarization. This construction would enhance perception of contrast.

This work was supported by the Grass Foundation and an NIMH Training Grant to the Department of Psychiatry at the University of California, San Diego, Medical School.

Circadian rhythm of photoreceptor cells in the Limulus *lateral eye: further studies.* TAKEHIKO SAITO, EHUD KAPLAN, AND ROBERT B. BARLOW, JR.

The sensitivity of the *Limulus* lateral eye undergoes a circadian rhythm. At night a clock in the brain transmits nerve impulses via efferent fibers to the ommatidia of the compound eyes. The efferent input increases the amplitude of the photoreceptor response to light and decreases the frequency of discrete potential fluctuations in the dark (*Nature,* **286**, 393–395, 1980).

We report here that cutting the optic nerve at night reverses the action of the circadian clock on the photoreceptors. After nerve section the frequency of the spontaneously occurring discrete waves increases and the amplitude of the receptor potential decreases. The signal-to-noise ratio of the retinular cells is thereby reduced. No consistent changes in cell resting potential were detected after nerve section. Spontaneously occurring nerve impulses recorded from eccentric cells *in situ* were triggered by discrete fluctuations in membrane potential; impulses caused by injury (nerve section) were not. The potential fluctuations of eccentric cells arise from discrete waves of retinular cells via electrical coupling of the retinular cells to eccentric-cell dendrite. The origin of spontaneous optic nerve activity *in situ* can thus be traced to the photoreceptors.

Under conditions of normal clock input, the decline of photoreceptor noise precedes the increase of retinal sensitivity. In the early evening hours when the endogenous efferent activity begins, the frequency of spontaneously occurring discrete waves of the photoreceptors decreases to a low level before the amplitude of the ERG increases significantly. This result is consistent with our previous finding, which indicates that separate cellular mechanisms underlie the clock-induced changes in the signal and noise characteristics of the photoreceptors.

Supported by NIH grants EY-00667, EY-00108, EY-00188, and NSF grant BNS 77-19436.

An optical determination of the resistance in series with the axolemma of Loligo pealei. B. M. Salzberg, F. Bezanilla, and H. V. Davila.

The membrane capacitance in squid giant axons has, in series with it a small resistance arising primarily in the narrow Schwann cell clefts. The true transmembrane potential therefore differs from that recorded between voltage electrodes in a voltage clamp by an amount proportional to the membrane current. If this resistance is not properly compensated, serious errors are introduced into membrane conductance and AC impedance measurements.

We have used an optical method to measure the series resistance by exploiting the potential-dependent changes in light absorption exhibited by an axon stained with a merocyanine-oxazolone dye, NK 2367. This dye behaves as a linear potentiometric probe with a microsecond time constant. The transmission at 720 ± 15 nm of a perfused voltage clamped giant axon, mounted on the stage of a compound microscope, was recorded by a silicon photodiode positioned in the objective image plane during hyperpolarizing and depolarizing potential steps (∓ 70 mV from -70 mV). The optical record during the hyperpolarization closely resembled the voltage clamp step, since no ionic current crosses the series resistance. During the depolarizing step, however, the transmitted intensity revealed a component proportional to the membrane current and series resistance. The feedback compensation (Hodgkin, Huxley, and Katz, 1952) was then varied until the optical signal assumed a square shape during both the hyperpolarizing and depolarizing potential steps. The value of the series resistance could then be read from a calibrated potentiometer in the feedback circuit.

In natural seawater, the value of the series resistance obtained in this manner was 2.8 ± 0.6 ohm cm^2. The method described here is not essentially electrical, but employs a 15 Å molecular voltage probe to afford an independent determination of the series resistance. Because the probe is located between the axolemma and the external series resistance, it is capable of distinguishing between the effects of the series resistance itself, and the anomalous dispersion of the membrane dielectric.

This work is dedicated to Kenneth S. Cole on the occasion of his 80th birthday, and was supported by NSF grant BNS 77-05025 and PHS grants AM 25201 and NS 12253.

The birefringence response of voltage-clamped internally perfused axons. Virginia Scruggs and David Landowne.

The birefringence of squid axons was measured by placing the axon between crossed polarizing crystals and measuring the transmitted light. When the membrane potential was changed from -10 to -140 mV the birefringence of the axon increased by about 10^{-4}. The birefringence response to a pulse from -70 to 0 mV was smaller and rose more slowly when compared with the response to a hyperpolarizing pulse.

Pairs of pulses were used to compare the birefringence response to a depolarizing pulse with and without sodium inactivation. When the records with a conditioning pulse to -140 or to 0 mV were made to coincide at the beginning of the second pulse they diverge after an initial fast phase. After the first 100 μsec the response corresponding to the inactivated sodium current has a smaller slope.

Both of these assymmetries in the birefringence response were also seen with symmetrical pulses with the depolarizing pulse beyond the sodium equilibrium potential, and also in the presence of tetrodotoxin, demonstrating they are not simple artifacts produced by current flow.

Suported by NIH research grant NS 13789.

Arsenazo III reveals long-lasting intracellular calcium transient following the action potential in squid giant presynaptic terminal. Stephen J Smith, Milton P. Charlton, and Robert S. Zucker.

The calcium indicator dye arsenazo III was used to measure cytoplasmic calcium concentration transients in presynaptic terminals of the stellate-ganglion giant synapse of *Loligo pealei.* Terminals were filled with arsenazo by passing a steady current from a dye-filled micropipette inserted near the tip of the terminal digit. Light absorption by the dye was measured by focusing filtered light from a tungsten-halogen source on the presynaptic terminal, and conducting transmitted light to a photodiode using a light pipe.

Individual presynaptic action potentials elicited rapid changes in light transmittance with the spectral properties expected from an increase in calcium binding to the dye. A maximal transmittance decrease was observed at 660 nm, a smaller decrease at 610 nm, and no change at 578 or 690 nm. The optical signal reached a peak in about 1 msec beginning coincident with the peak of the presynaptic action potential. Recovery of baseline optical transmittance was very slow, requiring approximately 20 sec at 15°C. Action potentials elicited repetitively over a wide range of intervals always gave incremental signals of similar amplitude. At high dye concentrations, a marked suppression of synaptic transmission was usually observed but good optical signals were obtained by signal averaging at low dye concentrations, where transmission appeared essentially normal.

Detecting calcium ions in cytoplasm at this short latency supports the currently accepted view that transmitter release is triggered by such a calcium transient. The very slow time course of dye signal recovery compared to transmitter release termination probably reflects spatial redistribution of calcium ions in the cytoplasm following entry at the surface membrane. The detection of residual free calcium long after the action potential is consistent with earlier suggestions that residual calcium may underlie certain aftereffects of transmission such as presynaptic facilitation.

S.J.S. and M.P.C. are Steps Toward Independence Fellows. R.S.Z. supported by a Sloan Foundation Fellowship and NIH grant NS15114.

Calcium and potassium activities in the hemolymph of the squid, Loligo pealei.

RODERIC E. STEELE AND DANIEL L. GILBERT.

Although the Ca and K concentrations in the hemolymph of the squid, *Loligo pealei,* have been previously measured, the activities of these ions have not. Physiological functions presumably depend upon the chemical activities, rather than the concentrations. Activity ratios were measured using microelectrodes (2–5 μm) filled with Ca^{2+} exchanger (WP Instruments, New Haven, CT) or K^+ exchanger (Orion Research, Cambridge, MA) and saturated KCl reference electrodes. For K^+, a double well wax sample holder was used to prevent K^+ leakage from the reference electrode into the sample being measured. The activity ratio of the Ca^{2+} in the hemolymph to the Ca^{2+} in artificial sea water (9.27 mM $CaCl_2$, 9.0 mM KCl, 423 mM NaCl, 22.9 mM $MgCl_2$, 25.5 mM $MgSO_4$) was 1.05 ± 0.02 (SEM) ($n = 9$ squid). For K^+, this ratio was 1.21 ± 0.04 (SEM) ($n = 11$ squid). Using activity coefficients (Smith, CRC Handbook of Marine Science, Vol. I, CRC Press, Cleveland, 1974), the calculated activities in millimoles/kg H_2O are 2.0 for Ca^{2+} and 6.8 for K^+. When compared to the natural sea water in which the squid were swimming, the activity ratios become 0.99 ± 0.02 (SEM) for Ca^{2+} and 1.26 ± 0.08 (SEM) for K^+. However, Requena *et al.* (1977, *J. Gen. Physiol.* **70**: 329) found that the Ca^{2+} concentration in the external media had to be lowered to 3 mM (corresponding to a concentration ratio of about 0.3) in order for the excised squid nerve to be in a steady state for Ca. Perhaps this difference is due to a change in the active transport or permeability for Ca in the excised nerve. The K^+ activity ratio of 1.26 can be compared with molal concentration ratios of hemolymph/sea water of 2.22 (Robertson, 1965, *J. Exp. Biol.* **42**: 153), 1.94 (Hodgkin, 1958, *Proc. Roy. Soc. B. Biol.* **148**: 1), 1.51 (Manery, 1939, *J. Cell. Comp. Physiol.* **14**: 365), and 1.34 (Shoukimas *et al.*, 1977, *Biophys. J.* **18**: 231). K^+ activities were elevated by 20–50% in 3 samples, which were taken 2 min after the initial sample, presumably due to K^+ leakage from cells. Such a leakage could explain the higher K^+ values found by some other investigators.

We wish to thank Kenneth S. Cole for stimulating this investigation.

Elimination of synapses from identified lobster motor neurons during development.

PHILIP J. STEPHENS AND C. K. GOVIND.

During development, inherent limitations are imposed on the peripheral fields of motor neurons. However, the mechanisms that impose such peripheral-field limitations are poorly understood. We have categorized the peripheral fields of two identifiable lobster motor neurons during normal development.

Pairs of deep extensor muscles are located in each abdominal segment, and in each hemisegment the muscle is divided into medial (DEAM) and lateral (DEAL) bundles. The second root nerve carries six motor axons to the deep extensor muscle in each hemisegment. The common excitor (CE) and common inhibitor (CI) motor axons in each root nerve can be identified at the periphery since they are the only axons that innervate both the DEAL and the DEAM.

In abdominal segments 2–5 of embryonic lobsters the CE and CI axons make functional connections with deep extensor muscle fibers in their own segment and in adjacent anterior and posterior segments. Junctional activity was not produced via central pathways and there was no detectable electrical coupling between muscle fibers in adjacent segments. Therefore the CE and CI motor axons must branch and make synaptic connections with deep extensor muscle fibers in three abdominal segments.

This innervation pattern was found for the CE and CI axons in embryonic, larval, and early juvenile lobsters. However at juvenile stage 6 the CE and CI connections were confined to the deep extensor fibers in their own segment. This is the typical distribution of CE and CI synapses in the adult.

Our results suggest that one way for a motor neuron to define its peripheral field involves an initial over-production of synapses followed by selective elimination.

Supported by a Grass Fellowship (P.J.S.), NIH-NINCDS and the Muscular Dystrophy Association of Canada (C.K.G. and H. L. Atwood).

Gap junctions: quantitative comparison of reduction in conductance by H and by Ca ions in an internally perfused preparation. J. H. Stern, D. C. Spray, A. L. Harris, and M. V. L. Bennett.

Conductance of gap junctions between *Ambystoma* and *Fundulus* blastomeres is decreased at low intracellular pH. The relation is well fit by a Hill plot with $K_H \sim 50$ mM ($pK_H \sim 7.3$) and Hill coefficient of 4.5 (Spray, Harris, and Bennett, *Science*, in press). To assess the relative effect of calcium ions we perfused one cytoplasmic face of the junctions with defined and rapidly changeable solutions. One cell of a coupled pair of *Fundulus* blastomeres ($\sim 100\ \mu$ diameter) was sucked into an internally perfused pipette ($\sim 20\ \mu$ internal diameter) until the second cell sealed against the pipette. The first cell was broken and its cytoplasm washed away by perfusate, leaving a membrane patch which included gap junctions. With current applied between bath and pipette interior, the ratio of voltages across the outer membrane of the intact cell and the patch gave the ratio of conductances of the patch (g_p) and outer nonjunctional membrane (g_n). Changes in g_p/g_n were presumably largely in junctional membrane since perfusion of a patch of a single cell had little effect. Perfusion solutions contained 100 mM KCl, 5 mM NaCl, 10 mM HEPES, and 10 mM EGTA or NTA. $CaCl_2$ and $MgCl_2$ were added to obtain 1 mM free Mg^{2+} and desired levels of buffered Ca^{2+}, confirmed with Ca^{2+}-sensitive macroelectrodes.

Changes in g_p/g_n with perfusates at pCa 7.0 and pH 6.8, 7.2, and 7.8 were rapid, reversible, and consistent with our previous measurement on intact cells. g_p/g_n was essentially unchanged by perfusates at pH 7.8 and pCa 7.0–4.0 but was progressively reduced by perfusates at pCa 3.9–3.0, with an apparent $K_{Ca} \sim 0.4$ mM ($pK_{Ca} \sim 3.4$). Thus junctional conductance is insensitive to cytoplasmic free Ca^{2+} concentrations below 0.1 mM (pCa 4) whereas junctional conductance is profoundly depressed by 0.1 μM hydrogen ion (pH 7).

External K^+ and Rb^+ retarded closing of potassium channels. R. P. Swenson, Jr., and C. M. Armstrong.

Gating properties of ionic channels have previously been considered to be insensitive to changes in the rate or species of ions moving within them. For example, Hodgkin and Huxley (1952) reported no change in Na channel kinetics when external Na concentrations were lowered, and Chandler and Meves (1965) found identical Na channel kinetics with either Na^+ or K^+ as the current carrier. In contrast to the immunity of channel gating to ions moving through the pore; blocking cations, *e.g.* local anesthetics, which bind within the Na channel, can prevent the Na channel from closing.

In contrast to previous results, we have found the closing kinetics of K channels to be slowed following equimolar substitution of extracellular Na^+ by either K^+ or Rb^+. Using a double pulse protocol to avoid complications arising from changes in K^+ concentrations in the confined extracellular space near the membrane, we were able to accurately measure K channel closing kinetics. At −70 mV the time constants were 3.0, 4.5, and 6.5 msec in artificial sea water (ASW), 200 K^+-ASW, and 200 Rb^+-ASW respectively at a temperature of 8° C. Instantaneous I–V relations in the same solutions suggest that external K^+ and Rb^+ enter the channel equally well, but Rb^+ remains in the channel about five times longer at inside negative potentials. The longer dwelling time of Rb^+ in the K channel than the K^+ itself correlates well with the slower rate of closing when Rb^+ is present externally.

When blocking Ba^{2+} ion binds near the external end of the potassium channel, K channels close with normal kinetics, but binding of TEA^+ near the inner end of the channel slows closing. In light of these observations, we suggest that a cation, permeant or not, at a site near the inner end of the channel is responsible for the retarded closing of potassium channels.

This work was supported by an NIH post-doctoral fellowship NS 06201-01 to R.P.S. and NIH NS12547 to C.M.A.

Swelling of squid giant axon during action potentials. I. TASAKI AND K. IWASA.

Rapid pressure changes and surface displacements in the squid giant axon were demonstrated during action potentials. To measure pressure changes, a Gulton piezo-ceramic bender was used in conjunction with a voltage follower. For detecting pressure changes, a Fotonic sensor placed about 0.1 mm above a small piece of gold leaf on a giant axon was employed. The output of the Gulton bender or of the Fotonic sensor was amplified with a capacity-coupled amplifier with a gain of 1000 and was led to a signal averager. The time course of the mechanical response of the axon observed by these two methods was diphasic. The first phase was found to coincide with the depolarizing phase of the action potential and to represent swelling of the axon. The magnitude of the outward displacement of the axon surface at the peak of swelling was about 1 nm and the corresponding pressure rise was, on the average, 10 μg per 0.3 mm^2. The second, shrinkage phase of the mechanical response started roughly at the onset of the hyperpolarizing after-potential: at its peak the magnitude of the surface movement observed was 1–4 nm and that of the pressure change was 10–50 μg per 0.3 mm^2. The mechanical responses associated with action potentials induced by lowering the external Ca-ion concentration were very similar to those evoked by electrical stimulation. External application of ouabain (up to 1 mM) or trypsin (1 mg/ml) had no clear effect on the mechanical responses for more than 1 hr after application; this finding suggests that Schwann cells outside the axon play little or no role in producing mechanical responses. The present findings indicate that action potential production is accompanied by drastic changes in the macromolecules in or near the axolemma. Many aspects of the experimental results are consistent with the old theories of excitation proposed by Loeb, Höber, Teorell, and others.

Presynaptic calcium currents and facilitated transmitter release in the giant synapse of Loligo pealei. ROBERT S. ZUCKER, MILTON P. CHARLTON, AND STEPHEN J SMITH.

At moderate levels of transmission, the giant synapse in the stellate ganglion of the squid exhibits facilitation, such that the second of two EPSPs (excitatory postsynaptic potentials) is larger than the first. We tested the possibility that synaptic facilitation is caused by a larger calcium influx during the second presynaptic impulse.

We measured the current carried by calcium ions using a three-electrode voltage clamp of the presynaptic terminal. Sodium currents were eliminated by external tetrodotoxin (2×10^{-7} g/ml) and potassium currents were blocked by 2 mM 3,4-diaminopyridine and intracellular injection of tetraethylammonium. Leak and capacitative currents were reduced and calcium currents smoothed by averaging the currents accompanying several equal and opposite pulses. When two spike-like depolarizing pulses (50 mV amplitude, 1 ms duration), giving a moderate level of transmitter release (5 mV EPSPs), were separated by 3 ms, the second EPSP was about 50% larger than the first. Both pulses were accompanied by a cadmium-sensitive inward calcium current of about 40 nA in the end terminal region.

In most experiments the currents during the two pulses were identical. In a few preparations, the peak inward current during the second pulse appeared about 5% larger than that accompanying the first. When a single depolarizing pulse was increased in amplitude or duration to give an EPSP equal to the facilitated EPSP, the calcium current was increased by 12–25%. We conclude that calcium channels facilitate very little if at all, and that facilitation of calcium influx cannot be primarily responsible for facilitation of transmitter release at this synapse.

Supported by NIH grant NS15114. R.S.Z. is an Alfred P. Sloan Research Fellow and M.P.C. and S.J.S. are Steps Toward Independence Fellows.

PARASITOLOGY AND IMMUNOLOGY

Solid-phase radioimmune assay to detect antibodies to Entamoeba histolytica. Sergio Arias-Negrete, Dianne McMahon-Pratt, Willy F. Piessens, and Ian Rosenberg.

We describe a solid-phase radioimmune assay for detecting antibodies to *Entamoeba histolytica* strain HM-1 cultivated axenically in TYI-S-33 (trypticase, yeast extract, iron and serum) media. To prepare the *E. histolytica* antigen, the amebas were pooled and centrifuged for 3 min at 500 × *g*, and washed three times with phosphate buffered saline (PBS) at pH 7.2. The trophozoites were disrupted ultrasonically and the insoluble material removed by centrifugation for 5 min at 12,000 × *g*. Phenylmethylsulfonylfluoride and Aprotinin were added to a final concentration of 2 mM and 0.5 units, respectively. The antigen was stored at −70°. Five female mice BALB/cJ were injected intraperitoneally with 75,000 trophozoites. On Day 14 post-inoculation, the animals were bled from the retro-orbital sinus. The serum was heated 30 min at 56°C. Polyvinylchloride plates were coated with 4–5 μg of *E. histolytica* antigen in 0.05 ml of PBS and incubated overnight at 4°C. The plates were then washed five times with PBS containing 4% fetal calf serum. Serial dilutions of 0.05 ml samples were made in the plates and incubated 6–7 hr at 4°C. To detect mouse anti-ameba serum bound to the plates, 0.04 ml of ^{125}I-rabbit anti-mouse IgG were added and incubated for 1 hr in an ice bath. The plates were washed and the samples counted in a gamma counter. The results showed that mouse anti-*E. histolytica* serum specifically bound the antigen as compared to the normal mouse serum. This antigen-antibody reaction is time- and temperature-dependent. This assay is more sensitive than enzyme-linked immunosorbent assay in detecting anti-ameba antibodies produced during the primary immune response to *E. histolytica.*

Inflammation in the sea star, Asterias forbesi. F. B. Bang.

Inflammation is a response of living tissue to local injury, leading to a local accumulation of blood cells and fluid. Following the injection of foreign pigmented cells of the sea urchin *Arbacia* into small (6–10 gm) sea stars, plugs of phagocytosed *Arbacia* cells accumulated within the transparent papulae of the skin of the sea stars. During the early phase (8–24 hr) these cells migrated through the papulae, often producing holes in the papular wall. A change of permeability of the papulae was detected after 4 hr by immersing the star in an 0.1% seawater solution of Evans blue dye for 20 min. This change in permeability disappeared by 48 hr with beginning resolution of the lesion. Not all of the urchin cells were carried out of the otherwise normal sea stars in this way; many were digested, and brownish pigment persisted in the papulae for a week or more. Inert material such as carmine powder is also known classically to be disposed of by penetration, but large amounts of carmine are found circulating within the amebocytes of the coelom weeks after injection. The inflammatory process induced by urchin cells spread to the separate water vascular system, especially after several injections of urchin cells into the coelom, as shown by marked clumping of amebocytes within the antenna and feet of the star. Edema, consisting of a ballooning of the epithelium, especially around the spines and paxillae of the skin, occurred usually after several injections of urchin cells, often in the injected limb, and lasted for several days. About 50 individual stars have been followed through this sequence of changes, and study of the influence of external variables on the response has been initiated.

Supported by NIH 5 P50 HL 19157.

Bacterial kidney disease of rainbow trout in sea water. James C. Carlisle and Jan Spitsbergen.

To investigate the influence of sea water acclimation on the pathogenesis of kidney disease (KD) in rainbow trout, *Salmo gairdneri*, 16 juveniles were infected on day 0 by intraperitoneal injection of 10^9 *Renibacterium salmoninarum*, the causative agent, and 16 were sham injected. Each group was subdivided into a fresh water group and a group to be exposed to gradually increasing salinity to reach full strength by day 31. Each of the four groups was kept in a 20-gal aerated and filtered aquarium at 15°C with 20% of the water replaced daily. Dissolved O_2 remained above 9 mg/l, NH_4 below 0.1 mg/l, and NO_2 below 0.3 mg/l.

A fish from each group was skin tested weekly by injecting 5×10^7 *R. salmoninarum* into the adipose fin, then bled and killed 1 week later. Humoral and cellular immunity were evaluated by bacterial agglutination, indirect fluorescent antibody test, and leukocyte migration inhibition. Spleen cells were tested for their ability to bind antigen.

Total mortality in the sea water infected group was 55% with a mean of 11.6 days between infection and death. In fresh water, mortality was 57.2% with a mean of 24 days until death. Among the uninfected fish in sea water, 22.2% died after an average of 32 days in increasing salinity. No fresh water control fish died.

Skin tests did not demonstrate grossly visible delayed type hypersensitivity. Infected fish showed lymphocyte-mediated migration inhibition on days 35 and 42.

Humoral antibodies as measured by bacterial agglutination were present in infected fish on days 14, 21, and 28, and in one control fish on day 28. Antibody activity measured by indirect immunofluorescence was present in infected trout on days 28 and 35, and in control trout on day 28. On the 28th day all fish had been skin tested 1 week previously, while on day 35 the controls had not been skin tested.

Antigen binding cells were present in the spleens of infected trout on days 21 and 28.

Schistosomiasis immune evasion. FRANCIS W. KLOTZ, SARA ANDERSON, SERGIO ARIAS-NEGRETE, DAVID KOECH, BARBARA SHERRY, AND ALAN SHER.

Lung forms of *Schistosoma mansoni* acquire host antigens and are not recognized by the immune system as foreign. We investigated this immune evasion by lung schistosomula of *S. mansoni.* To determine the sites of immune effector mechanisms and the efficacy of immunization against schistosomula, we infected inbred mice with *S. mansoni* cercariae skin-derived schistosomula, and lung-derived schistosomula. Cercariae collected from *S. mansoni*-infected *Biomphalaria glabrata* were irradiated with a 20 Krad dose of X rays from a ^{60}Co gamma source. Thirty C57BL/6J female mice were each exposed percutaneously to 500 X-irradiated cercariae. Six weeks later these mice and 30 age-matched controls were challenged with 200 normal cercariae percutaneously, 200 skin-derived schistosomula intravenously or 5-day-old lung-derived schistosomula intravenously. The lung schistosomula were derived from syngeneic, age-matched donors. Four weeks post-challenge, the mice were killed and the adult worms recovered by perfusion from the hepatic portal system. We recovered 5 ± 6 adult worms from the immunized, cercaria-challenged animals compared to 71 ± 27 from the controls for 93% protection. We recovered 10 ± 7 adults from the immunized, skin-schistosomula-challenged animals compared to 83 ± 10 from the controls, indicating 88% protection. We recovered 6 ± 7 adults from immunized, lung-schistosomula-challenged mice compared to 67 ± 18 from the controls, indicating 91% protection. The data is significant at the 99% confidence limit. Immune evasion by lung schistosomula did not occur in this preliminary experiment.

A natural protozoan-derived ionophore: a possible mechanism of cytotoxicity by Entamoeba histolytica. EILEEN LYNCH, ANDY HARRIS, IAN ROSENBERG, AND CARLOS GITLER.

Using the technique of planar lipid bilayers, we have reconstituted a natural ionophore channel from extracts of the pathogen *E. Histolytica.* Both the conditioned media in which amoebas had been grown and cell homogenates of the organism exhibited spontaneous channel-forming activity, whereas control media did not. Addition of microgram quantities of extract to the bilayer results in stepwise increases in membrane conductance equal to 1600 pmhos in 1 M KCl. Such increments occur at a linear rate to increase membrane conductance up to 5 orders of magnitude. Ion channels so induced display a moderate cation selectivity with the permeability ratio of potassium to chloride = 4.6 and that of sodium to chloride = 2.5. The amoeba pore exhibits a time variant, voltage dependent behavior with both negative and positive values of transmembrane field decreasing membrane conductance. More than 75% of the induced conductance is inactivated by protease addition to the system. Additionally, under recording conditions, we show that microgram quantities of amoeba extract irreversibly decrease the input membrane resistance of an impaled *Fundulus* blastomere 1.12 mega-ohms. Such a resistance change corresponds to a conductance increase of 630 nmhos; such an increase would be produced if 4000 amoeba channels were inserted across the blastomere membrane.

These data demonstrate that extracts of *E. Histolytica* can impose striking changes upon the membrane permeability of both living and artificial systems. Furthermore, these data suggest that the *in vivo* mechanism of cytotoxicity and pathology induced by the amoeba may operate through the insertion of parasite-derived channels into the host cell membrane.

This work was supported in part by NIH Training Grant T 32 GM-7288, and the M.B.L.

Labeling of microfilariae of Brugia malayi. WILLY F. PIESSENS, IAN ROSENBERG, CARLOS GITLER, AND STUDENTS OF THE BIOLOGY OF PARASITISM COURSE.

The antigens that play a role in the biologic interaction between microfilariae of *B. malayi* and the infected host remain to be identified. To identify the antigens, we developed methods to label parasite materials with lectins and by iodination procedures. Of seven lectins tested, only wheat germ agglutinin bound to the sheath of microfilariae, in a pattern that appeared linear and homogeneous by direct immunofluorescence. Microfilariae could also be labeled with ^{125}I by the lactoperoxidase, Bolton and Hunter, iodogen, and iodonaphtylazide techniques. Each labeling method produced a different banding pattern on linear SDS-polyacrylamide gradient gels of deoxycholate extracts of labeled microfilariae. A single band (mol. wt. 67,000 daltons) was labeled by all four methods. Whether any of the labeled materials can be adsorbed onto wheat germ agglutinin-sepharose columns or precipitated by immune serum remains to be determined.

Monoclonal antibodies to Leishmania enriettii *and tubulin.* D. MCMAHON PRATT AND STUDENTS OF THE PARASITOLOGY COURSE.

Köhler and Milstein demonstrated in 1975 that by fusion of myeloma cells with spleen cells from immunized animals, cloned hybrid cell lines could be produced which secrete antibodies specific for the immunizing antigen. Such monoclonal antibodies can potentially be of great use in the diagnosis and treatment of parasitic diseases. In the Parasitology Course at Marine Biological Laboratories this summer, as a class experiment, monoclonal antibodies to the protozoan parasite *Leishmania enriettii* were produced. The experimental procedure was as follows: Balb/c mice, immunized with membranes from *L. enriettii,* were boosted with antigen. Three days later the spleens from these animals were removed and fused with cells of the myeloma cell line NS-1 using polyethylene glycol 1000. The fused cells were cultured in 96 well microtiter plates. Hybrids were selected by growth in HAT (hypoxanthine-aminopterin-thymidine) medium. Of the total 2612 wells cultured, hybrid cells grew in 904 wells. After 14 days of culture, antibody production by the hybrid cells was evaluated using a solid phase radioimmune assay. Two hundred and sixteen wells were determined to be secreting antibody. Selected antibody producing wells were cloned by limiting dilution on a feeder layer of x-irradiated (2500 rads) mouse spleen cells. Two weeks later the cloned cells were evaluated for antibody production. Of the initial 42 antibody producing wells, 19 resulted in cloned antibody producing hybrids. Several of these monoclonal antibodies to *L. enriettii* are being further characterized with respect to both the subclass of immunoglobulin and the *Leishmania* antigen recognized. Of these, one monoclonal antibody has been demonstrated specific for tubulin, a major membrane protein constituent of *Leishmania.* The identification of the tubulin specific monoclonal antibody was done in collaboration with Marcel Hommel and Mary Porter.

Stages in the life cycle of the digenetic trematode, Lasiotocus minutus *(Manter, 1931) Thomas, 1959.* HORACE W. STUNKARD.

Lasiotocus minutus, a parasite in the intestine of *Menidia menidia,* was described and named by Manter (1931) from specimens taken at Beaufort, North Carolina. It is a member of the Monorchiidae, a large family with 12 subfamilies, about 30 genera, and 100 species, which infect marine fishes in all parts of the world. Observations on different stages in the life history of certain species have been recorded and the complete life cycle of a single species, *Monorcheides cumingiae,* was worked out by Martin (1940) at the M.B.L. The 1959 paper by Stunkard and Uzmann (*Biol. Bull.* **116**: 184–193) on the life cycle of *Proctoeces maculatus* included the description of a microcercous cercaria, designated as *Cercaria adranocerca,* found in *Gemma gemma* collected at Boothbay Harbor, Maine. During the summer of 1980, examination of specimens of *G. gemma* taken in the Woods Hole area yielded infections with *C. adranocerca* and study has elucidated the life cycle of the species. The cercariae, which proved to be the larval stage of *L. minutus,* are microcercous and cannot swim.

Soon after their emergence from sporocysts, they become encysted in the haemocoele of the clam. They may be extruded singly or in strands embedded in a jelly-like matrix. The metacercariae were fed to small fishes and a series of developmental stages was recovered from the silversides, *Menidia menidia*. No infections were established in *Fundulus heteroclitus* or 0-year *Pseudopleuronectes americanus*. Revisions of classification of monorchiid trematodes have resulted in many changes and the species long known as *Genalopa minuta* is now recognized as *Lasiotocus minutus* (Manter, 1931) Thomas, 1959.

Investigation supported by NSF-DEB 80-06150.

Purification of parasite mRNA. D. Wirth, R. Carter, L. Miller, and M. Hommel.

To identify stage specific mRNA in two parasite systems analyzed, total mRNA was purified from each parasite and translated in a cell-free system. The products were analyzed on protein gels. In *Plasmodium gallinaceum*, the gamete and asexual stage RNAs were isolated. There appear to be several parasite-specific proteins in the translation products of RNA isolated from gametes and these proteins will be analyzed using monoclonal antibodies raised against gametes. The RNA isolated from *L. enriettii* amastigote stage directs the synthesis of many proteins and these need to be identified using specific antisera.

Tubulin genes in parasites. D. Wirth, C. Gitler, and I. Rosenberg, (in collaboration with C. French, D. Mischke, and M. Pardue).

Two parasites examined, *Trypanosome brucei* and *L. enriettii* were known to contain tubulin; no tubulin had been detected in the third parasite, *Entamoeba histolytica*. To detect tubulin genes in parasites, DNA was purified from each parasite, cut with a restriction enzyme (Eco RI) and fractionated on an agarose gel. The fractionated DNA was transferred to nitrocellulose and hybridized to a radiolabeled tubulin gene isolated from *Drosophila*. The *Drosophila* tubulin gene clearly hybridized to a single EcoI fragment of *L. enriettii* DNA and to two EcoI fragments of *T. brucei* DNA. No hybridization to *Entamoeba histolytica* DNA was seen in the first experiment and weak, possibly non-specific hybridization was found in a second experiment.

Cloning genes of Leishmania enriettii *in* E. coli. D. Wirth, Dianne McMahon Pratt, John David and students in the Biology of Parasitism Course.

The goal of this experiment was to make a genomic library of *L. enriettii* DNA and to detect expression of parasite-specific antigens in *E. coli*. If such expression did occur, antigens suitable for vaccination might be produced in bacteria. Total DNA was purified from *L. enriettii* promastigotes, cut with the restriction enzyme PST I and ligated to the plasmid pBR322, which had also been cut with PST I. *E. coli* bacteria were transformed with these recombinant plasmids and the transformed bacterial clones selected for growth in the presence of tetracycline, a drug resistance to which was encoded by the plasmid. Approximately 1000 transformed *E. coli* clones arose per microgram of recombinant plasmids. Of the transformed clones 82% contained a segment of *L. enriettii*, as indicated by the sensitivity of the clones to ampicillin. The transformed *E. coli* clones were screened for the expression of parasite antigens using a solid phase radioimmunoassay. The antiserum used in this assay was raised against crude *L. enriettii* membranes and contained antibodies which react with several proteins. Five of 10,000 clones screened appeared to react with the antiserum. These clones have been isolated and will be retested.

PLANT PIGMENTS AND PHOTOSYNTHESIS

Colorless proteins in phycobilisomes of rhodophycean and cyanobacterial species. R. S. Alberte, T. A. Kursar, and R. F. Troxler.

Phycobilisomes are multimeric aggregates composed of phycobiliproteins which occur on the outer surfaces of thylakoid membranes. They function as light-harvesting antennae for photosynthesis in rhodophycean and cyanobacterial cells. Phycobilisomes contain allophycocyanin (APC; λ max. = 650 nm), phycocyanin (PC; λ max. = 620 nm) and in some species, phycoerythrin (PE; λ max. = 500, 545, 565 nm). Recently, it has been shown that phycobilisomes contain 8–10 colorless proteins in addition to the phycobiliproteins and it has been suggested that these colorless proteins function as structural components by linking the various phycobiliproteins to one another. In the present work, phycobilisomes from the unicellular rhodophyte, *Cyanidium caldarium*, the unicellular cyanobacterium, *Anacystis nidulans* (both producing APC and PC), were compared to those of the macrophytic red algae, *Neogardhiella* sp. and *Ceramium* sp. (both producing APC, PC, and PE).

C. caldarium phycobilisomes, not previously described, displayed a single absorption maximum at 624 nm (due to PC). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of *C. caldarium* phycobilisomes revealed approximately nine colorless proteins in the 24–75 kd range after staining with Coomassie Brilliant Blue. *A. nidulans* phycobilisomes displayed a broad absorption band (λ max. = 620 nm) due to PC, and SDS-PAGE revealed approximately nine colorless proteins in stained gels.

A new procedure was developed to enable isolation of phycobilisomes from macrophytic red algae. Phycobilisomes from *Neogardhiella* sp. and *Ceramium* sp. displayed absorption maxima at 498, 544, and 568 nm (due to PE), at 615 nm (due to PC) and at 650 nm (due to APC). Analysis of phycobilisomes from these species by SDS-PAGE revealed the γ subunit of PE (mol. wt. $\cong$ 31 kd) in addition to approximately nine bands ascribed to colorless proteins (after staining) in the 24–105 kd range. The pattern of colorless proteins on gels displayed minor differences among species and differed significantly with respect to the amount of protein (Coomassie Brilliant Blue staining) in a given M_r range.

The present study is the first to demonstrate the presence of colorless proteins in phycobilisomes of *C. caldarium*, *Neogardhiella* sp., and *Ceramium* sp. and provides a point of departure for demonstrating the function of the respective colorless proteins in attaching the various phycobiliproteins to one another.

Supported by NATO grant 1721, NSF PCM79 06638, and NIH GM 22822 and GM 23944.

Phycocyanobilin synthesis from exogenous heme. S. B. BROWN, R. F. TROXLER, AND R. S. ALBERTE.

In animals, biliverdin evidently is derived from protoporphyrin IX via the heme of hemoproteins. In plants, it is likely that, as with biliverdin, the carbon skeleton of phycobilins is derived from protoporphyrin IX, but it is uncertain whether ring cleavage occurs via a magnesium porphyrin derivative or via heme. Recently, we showed that the unicellular rhodophyte, *Cyanidium caldarium*, can take up heme from the suspending medium. This organism synthesizes chlorophyll-*a*, allophycocyanin, and phycocyanin when grown autotrophically but does not produce these pigments when grown heterotrophically. Photosynthetic pigments are synthesized when heterotrophic cells are placed in the light. When dark-grown (heterotrophic) cells were incubated with ^{14}C-heme and exposed to light, the phycocyanobilin chromophore was found to be ^{14}C-labeled, indicating conversion of heme to bile pigment by this organism.

We have repeated these experiments using the cyanobacterium, *Anacystis nidulans,* which unlike *C. caldarium*, cannot be grown heterotrophically but produces the same photosynthetic pigments (chlorophyll *a*, allophycocyanin, and phycocyanin). In the first experiment, 18 mg of ^{14}C-heme (11.7 dpm/mole) was added to 4500 ml of growth medium containing 225 ml inoculum of fully grown *A. nidulans* cells. After 6 days, growth was complete and the phycocyanobilin, cleaved from apoprotein and purified as the dimethylester, revealed little or no significant radioactivity. In a second experiment, addition of radiolabeled heme was delayed until cultures were grown to half their final cell density. The remaining growth occurred within 24 hr, after which phycocyanobilin dimethylester, obtained as before, again contained no significant radioactivity. In a third experiment, cells of *A. nidulans* in exponential growth were resuspended in nitrogen depleted medium, resulting in loss of phycocyanin and allophycocyanin. After nitrogen bleaching (36 hr), nitrogen and radiolabeled heme were added to the medium. When resynthesis of phycobiliproteins was completed, phycocyanobilin was again isolated as the dimethylester but it contained no radioactivity.

The failure of *A. nidulans* cells to convert ^{14}C heme to phycocyanobilin is probably due to the inability of these cells to take up heme from the suspending medium, although the possibility that heme might not be a precursor of phycocyanobilin in this organism cannot be discounted from the experimental results.

Supported by NATO grant 1721, NSF PCM79 06638, PCM78 20535, and NIH GM 22822.

Relationships between photosystem II and phycobilisomes in red algae and cyanobacteria. T. A. Kursar, D. Mauzerall, and R. S. Alberte.

The phycobiliproteins of the cyanobacteria and red algae are aggregated into complexes termed phycobilisomes (PBS). The PBS are localized on photosynthetic membranes and efficiently transfer excitation energy to reaction center II (RC-II). The ratio of chlorophyll (Chl) to RC-II was measured by the method of Emerson and Arnold, and the $Chl \cdot O^{-1} \cdot$flash number^{-1} was divided by four, the number of electrons necessary to make O_2. The values obtained were 330 (±25) Chl/RC-II for *Anacystis nidulans,* a cyanobacterium, and 320 (±25) Chl/RC-II for *Neoagardhiella baileyi,* a macrophytic red alga collected at Woods Hole. Filtration of algal homogenates through Celite removed essentially all of the Chl. The biliprotein concentrations in the filtrates were determined from specific extinction coefficients. The PBS of *Anacystis* and *Neoagardhiella* were shown to contain about 90% of the cellular biliprotein. If we assume that 10–15% of the PBS is composed of "uncolored proteins", the PBS-associated protein per RC-II was 3.0 and 3.7×10^6 daltons for *Anacystis* and *Neoagardhiella,* respectively. Based on published chromophore compositions of biliproteins, the total bilin chromophores per RC-II was 190 (±4) for *Anacystis* and 520 (±50) for *Neoagardhiella.* Molecular weights of isolated PBS were determined from S values obtained from sedimentation velocity measurements and diffusion coefficients measured by quasi-elastic light scattering (Paul Missel, MIT). *Anacystis* PBS were determined to be 3.6×10^6 daltons and *Neoagardhiella* PBS to be 12×10^6 daltons. Another PBS molecular weight was determined assuming a six-rod model for PBS structure, using literature data on tripartite particles (800 kd PBS building blocks from red algae), and using spectroscopically determined biliprotein compositions of *Anacystis* and *Neoagardhiella* PBS. The results yielded molecular weights of 5.1×10^6 and 14.1 (±2.0) daltons for *Anacystis* and *Neoagardhiella,* respectively. Consequently, the ratio of PBS per RC-II would be 1.45 (±0.25) for *Anacystis* and 3.4 (±0.5) for *Neoagardhiella.* It is possible that the RC-IIs in red algae are clustered into groups of three or four such that several RC-IIs share a single PBS.

Research supported by NSF grants PCM 77-09102, PCM 78-10535, and PCM 79–06638, and NIH grant GM-23944.

Photosynthetic light adaptation features of Zostera marina *L. (eelgrass).* L. Mazzella, D. Mauzerall, and R. S. Alberte.

The light adaptation features of photosynthesis in *Zostera marina* L. were studied in plants collected in a Woods Hole tidal bed. Our previous studies on *Zostera* showed a gradient in leaf pigmentation and photosynthetic activity along the leaf axes. Therefore, we sought to characterize in the laboratory the photosynthetic characteristics of *Zostera* leaves developed *in situ* under a range of light environments. Photon flux densities measured at the collection site (July, 1980) showed a gradient from the level of the tips of submerged plants to the bottom (2 m) (830–250 and 400–70 $\mu E \cdot m^{-2} \cdot sec^{-1}$ on cloudless and cloudy days, respectively). The maximal photosynthetic rates per unit area, measured as CO_2-dependent O_2 evolution, increased almost two-fold (3.0–5.6 $\mu Mol\ O_2 \cdot dm^{-2} \cdot min^{-1}$) from the leaf bases to the tips. However, when rates were expressed on a chlorophyll (Chl) basis, leaf bases had higher rates than tips (4.0 $\mu Mol\ O_2$ compared with 3.0 $\mu Mol\ O_2 \cdot mg\ Chl^{-1} \cdot min^{-1}$). The differences in Chl and area rates can be attributed to the fact that there is a 40% increase in Chl per dm^2 in leaf tips compared with bases. Light saturation of photosynthesis occurred 100 and 150 $\mu E \cdot m^{-2} \cdot sec^{-1}$ for all leaf types examined. Initial slopes of the photosynthetic light saturation curves, defined as photosynthetic efficiency, increased from leaf bases to tips when rates were expressed per unit area. Photosynthetic activities of young, old epiphytized, old non-epiphytized, and old leaves with epiphytes removed were compared along the leaf axes. In almost all cases photosynthetic activity increased from the bases to the tips. It is clear that epiphytes contribute a significant portion of the total leaf photosynthesis per unit area. Photosynthesis measured as $^{14}CO_2$-fixation rates paralleled the O_2 evolution rates described above. Such studies provide a molecular and biochemical framework for further investigations aimed at defining the adaptive physiology of a highly productive coastal marine species.

Research supported by NSF grants PCM 79-06638 and PCM 78-10535.

Photosynthetic activity of fungal infected and noninfected Laminaria saccharina *Lam.* SCOTT SCHATZ AND THOMAS A. KURSAR.

The sporophytic phase of the brown alga, *Laminaria saccharina* Lam., becomes susceptible to infection by the ascomycete, *Phycomelaina laminariae* (Rostr.) Kohlm. after 1 year of growth. Coincident with reduced rates of growth, diseased plants display a more limited photosynthetic capacity under saturating light conditions when compared with healthy plants. The base of the healthy blade tissue has a CO_2-dependent oxygen evolution rate of 1.1 μmoles $O_2 \cdot mg\ Chl^{-1} \cdot min^{-1}$ while the base of the blade of infected plants have rates of 0.44 μmoles $O_2 \cdot mg\ Chl^{-1} \cdot min^{-1}$. The rate of O_2 evolution in stipes, the site of infection was 0.08 μmoles $O_2 \cdot mg\ Chl^{-1} \cdot min^{-1}$ in healthy stipes and 0.01 μmoles $O_2 \cdot mg\ Chl^{-1} \cdot min^{-1}$ in infected stipes. The rate of respiration was found to exceed the net photosynthetic rate of infected stipes and may be attributable to increased rates of respiration due to the presence of the fungal pathogen. The functional organization of the photosynthetic apparatus, the photosynthetic unit (PSU), can be defined as the minimum number of chlorophyll molecules necessary to drive oxygen evolution. Interestingly, the PSU size in the base of blade tissue is similar in both healthy and infected plants (2100 ± 200 Chl $a+c$). The PSU size of the tip of the healthy blade was much greater (4500 Chl $a+c$). These data, taken together with previous PSU data on mid-blade tissues of healthy plants, indicate that as the blade of healthy plants ages there is a loss in the number of PSUs. It is possible that the decrease in photosynthetic rate in infected plants may be related to the numbers of functional PSUs or due to decreased activity of enzymes involved in the dark reactions of photosynthesis. It was not possible to determine whether the process of "whole plant senescence" led to increased susceptibility to infection, or whether fungal infection was the causal factor in decreased photosynthetic capacity.

Research supported by NSF grant PCM-79-06638 and PCM-780535.

*Chemical conversion of a chlorophyll-*a *derivative to a bile pigment.* K. M. SMITH, S. B. BROWN, AND R. F. TROXLER.

Little is known about the mechanisms of chlorophyll degradation in spite of the fact that millions of tons are removed from the biosphere annually during leaf senescence and grain ripening. In contrast, heme degradation to bile pigments is reasonably well understood and the origin of the bile pigment lactam oxygen atoms has been studied using ^{18}O labeling of molecular oxygen in a variety of systems.

Bile pigments have never been observed during photochemical chlorophyll breakdown *in vivo* or *in vitro*. Smith and coworkers have recently developed a chemical model reaction in which chlorophyll-*a*, after chemical derivatization, reacts with molecular oxygen to produce a bile pigment. In the present work, we have developed conditions whereby the reaction can be carried out in a closed system appropriate for ^{18}O labeling techniques used in heme degrading systems.

Zn(II)methyl-δ-mesotrifluoroacetoxypyropheophorbide-*a* was prepared by treating methylpyropheophorbide-*a* (derived from chlorophyll-*a*) with zinc acetate and thallium trifluoroacetate. We dissolved 5 mg of Zn(II)methyl-δ-mesotrifluoroacetoxypyropheophorbide-*a* in chloroform (5 ml) and added basic alumina (2–3 g). After shaking for 15 min in air, the reaction mixture was passed through a sintered glass funnel, the filtrate was evaporated to dryness, and the residue was applied to a silica gel G plate. The green mixture rapidly separated into two green bands, the upper (minor) corresponding to starting material and the lower (major) corresponding to ring-opened product. This quickly turned blue on the plate, presumably due to demetallation of the zinc complex of the bile pigment. The product was eluted with chloroform and evaporated to dryness yielding product as a residue. An identical result was obtained when the reaction was run in the absence of light indicating that it is not a photooxidation.

Using this method, it should be possible to carry out the reaction in a closed system under $^{18,18}O_2$. Mass spectrometry of the ring-opened product will reveal whether the lactam oxygen atoms are derived from the same oxygen molecule or from different oxygen molecules as in heme catabolism.

Supported by NATO grant 1721, NSF PCM79 06638, NIH GM 22822, NIH AM 25714, and a grant from the Nuffield Foundation.

*Synthesis of chlorophyll-*a *from δ-aminolevulinate in dark-grown* Cyanidium caldarium *cells.* R. F. TROXLER AND S. B. BROWN.

Autotrophic wild-type *C. caldarium* cells synthesize chlorophyll-*a*, allophycocyanin and phycocyanin but lack these photosynthetic pigments when grown heterotrophically in the dark. It was shown previously that dark-grown cells administered δ-aminolevulinate (ALA) in the dark excrete porphyrins and phycocyanobilin (the chromophore of allophycocyanin and phycocyanin) into the suspending medium. The cell pellet displayed a greenish-brown color, in contrast to the yellow color of non-ALA treated controls. The present work examined whether cells given ALA in the dark synthesize chlorophyll *a*.

Cells incubated with ALA were suspended in 50 mM Tris-HCl, pH 8, disrupted in a French pressure cell (20,000 p.s.i.) and centrifuged at 30,000 × *g* for 20 min. The greenish-brown pellet was suspended in 6 volumes of petroleum ether : acetone (5 :1, v/v) to which saturated NaCl (1/4 volume), water (1/4 volume) and diethyl ether (1 volume) was added. After shaking, the green ether phase was dried with Na_2SO_4, reduced to dryness, and the residue was applied as a band to silica gel G plates which were developed with petroleum ether : acetone (7 : 3, v/v). A green band was observed at $R_f = 0.66$ which chromatographed with authentic chlorophyll *a* prepared as above from autotrophic cells. The green band was eluted with 80% acetone and gave a spectrum identical to chlorophyll *a* with absorption maxima at 663, 618, 582, 536, and 433 nm.

The pellets from disrupted, ALA-treated cells were extracted with 0.1% sodium dodecyl sulfate and examined electrophoretically on 7.5% polyacrylamide gels. Complex I (P_{700} chlorophyll *a* protein) extracted from thylakoid membranes of autotrophic cells was absent in membrane fragments of cells incubated with ALA. Measurements on autotrophic cells indicated a photosynthetic rate of approx. 1 μl $O_2 \cdot min^{-1} \cdot mg\ chlorophyll^{-1}$ whereas O_2 evolution from cells incubated with ALA could not be detected.

The data indicated the *C. caldarium* cells synthesize chlorophyll *a* in the dark, that chlorophyll *a* is recovered in membranes possibly complexed with a precursor of P_{700} chlorophyll *a* protein, and that chlorophyll *a* synthesized in the dark is not photosynthetically competent.

Supported by NATO grant 1721, NSF PCM79 24139, PCM79 06638 and a grant from the Nuffield Foundation.

Continued from Cover Two

high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables, with their headings and footnotes, should be typed on separate sheets, numbered with consecutive Roman numerals, and placed after the Literature Cited. Figure legends should contain enough information to make the figure intelligible separate from the text. Legends should be typed double spaced, with consecutive Arabic numbers, on a separate sheet at the end of the paper. Footnotes should be limited to authors' current addresses, acknowledgments or contribution numbers, and explanation of unusual abbreviations. All such footnotes should appear on the title page. Footnotes are not normally permitted in the body of the text.

4. ***A condensed title*** or running head of no more than 35 letters and spaces should appear at the top of the title page.

5. ***Literature cited.*** In the text, literature should be cited by the Harvard system, with papers by more than two authors cited as Jones *et al.*, 1980. Personal communications and material in preparation or in press should be cited in the text only, with author's initials and institutions, unless the material has been formally accepted and a volume number can be supplied. The list of references following the text sould be headed LITERATURE CITED, and must be typed double spaced on separate pages, conforming in punctuation and arrangement to the style of recent issues of *The Biological Bulletin*. Citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS (BIOSIS List of Serials; the most recent issue). Foreign authors, and others who are accustomed to use THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

H. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond.; Science, N.Y.; Evolution, Lancaster, Pa.*)

6. ***Reprints, charges.*** *The Biological Bulletin* has no page charges. However, authors will be requested to pay printing charges in excess of $70 for reproducing tabular material, mathematical formulae, and figures. Reprints may be ordered at time of publication and normally will be delivered about two to three months after the issue date.

CONTENTS

ANDERSON, ROBERT S.
Hemolysins and hemagglutinins in the coelomic fluid of a polychaete annelid, *Glycera dibranchiata* 259

HADLOCK, ROBIN P.
Alarm response of the intertidal snail *Littorina littorea* (L.) to predation by the crab *Carcinus maenas* (L.) 269

HOUK, MARGARET S. AND RALPH T. HINEGARDNER
The formation and early differentiation of sea urchin gonads 280

KANESHIRO, EDNA S. AND RICHARD D. KARP
The ultrastructure of coelomocytes of the sea star *Dermasterias imbricata* 295

MARCUS, NANCY H.
Photoperiodic control of diapause in the marine calanoid copepod *Labidocera aestiva* 311

MATSUMOTO, GEN AND JUNICHI SHIMADA
Further improvement upon maintenance of adult squid (*Doryteuthis bleekeri*) in a small circular and closed-system aquarium tank 319

MCCORKLE, SUSAN AND THOMAS H. DIETZ
Sodium transport in the freshwater Asiatic clam *Corbicula fluminea*. 325

MERCANDO, NEIL A. AND CHARLES F. LYTLE
Specificity in the association between *Hydractinia echinata* and sympatric species of hermit crabs 337

MILLER, CHARLES B., DAVID M. NELSON, ROBERT R. L. GUILLARD, AND BONNIE L. WOODWARD
Effects of media with low silicic acid concentrations on tooth formation in *Acartia tonsa* Dana (Copepoda, Calanoida) 349

MORAN, WILLIAM M. AND RICHARD E. TULLIS
Ion and water balance of the hypo- and hyperosmotically stressed chiton *Mopalia muscosa* 364

OHTSU, KOHZOH
Electrical activities in the subtentacular region of the anthomedusan *Spirocodon saltatrix* (Tilesius) 376

RAHAT, M. AND ORIT ADAR
Effect of symbiotic zooxanthellae and temperature on budding and strobilation in *Cassiopeia andromeda* (Eschscholz) 394

SULKIN, S. D., W. VAN HUEKELEM, P. KELLY, AND L. VAN HUEKELEM
The behavioral basis of larval recruitment in the crab *Callinectes sapidus* Rathbun: A laboratory investigation of ontogenetic changes in geotaxis and barokinesis 402

TURNER, KATHERINE AND TIMOTHY A. LYERLA
Electrophoretic variation in sympatric mud crabs from North Inlet, South Carolina 418

YOUNG, CRAIG M. AND LEE F. BRAITHWAITE
Orientation and current-induced flow in the stalked ascidian *Styela montereyensis* 428

ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY 441

Volume 159 Number 3

Marine Biological Laboratory
LIBRARY
FEB 6 1981
Woods Hole, Mass.

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

DANIEL L. ALKON, National Institutes of Health and Marine Biological Laboratory
FREDERICK B. BANG, Johns Hopkins University
EDWARD M. BERGER, Dartmouth College
STEPHEN C. BROWN, State University of New York at Albany
HARLYN O. HALVORSON, Brandeis University
J. B. JENNINGS, University of Leeds
MEREDITH L. JONES, Smithsonian Institution
GEORGE O. MACKIE, University of Victoria
JOEL L. ROSENBAUM, Yale University
HOWARD A. SCHNEIDERMAN, Monsanto Company
F. JOHN VERNBERG, University of South Carolina
E. O. WILSON, Harvard University

Managing Editor: CHARLES B. METZ, University of Miami

DECEMBER, 1980

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $10.00. Subscription per volume (three issues), $27.00 (this is $54.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Editor; or Susan Schwartz, assistant editor, at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and at the Institute For Molecular and Cellular Evolution, University of Miami, 521 Anastasia, Coral Gables, Florida 33134 during the remainder of the year.

Copyright © 1980, by the Marine Biological Laboratory

Second-class postage paid at Woods Hole, Mass., and additional mailing offices.
ISSN 0006-3185

INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except for a limited number of solicited review papers which may be accepted after formal refereeing), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts, including figures, should be submitted in triplicate. (Xerox copies of photographs are not acceptable for review purposes.) The original manuscript must be typed in double spacing (*including* figure legends, footnotes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. Manuscripts should be proofread carefully and errors corrected legibly in black ink. Pages should be numbered consecutively. Margins on all sides should be at least 1 inch (2.5 cm). Manuscripts should conform to the *Council of Biology Editors Style Manual*, 4th Edition (Council of Biology Editors, 1978). Unusual abbreviations should be kept to a minimum and should be spelled out on first reference as well as defined in a footnote on the title page. Manuscripts should be divided into the following components: Title page, Abstract (of no more than 200 words), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, Tables, and Figure Legends. In addition, authors should supply a list of words and phrases under which the article should be indexed.

2. ***Figures.*** Figures should be no larger than 8½ by 11 inches. The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

3. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the

Continued on Cover Three

ERRATA

THE BIOLOGICAL BULLETIN, Volume **159**, Number 1, Pages 166, 167, 168.

Equations were printed incorrectly in the article by Keith Nelson, Dennis Hedgecock, Will Borgeson, Eric Johnson, Richard Daggett, and Diane Aronstein, "Density-dependent growth inhibition in lobsters, *Homarus* (Decapoda, Nephropidae" (1980, *Biol. Bull.*, **159**: 162–176). The equations should read as follows:

Page 166:

$$a_{\mathrm{j}} = a_0 + \sum_{\mathrm{l}=1}^{\mathrm{j}} \frac{r_{\mathrm{l}}}{vk} \qquad (2)$$

$$a_{\mathrm{j}} = (\mathrm{R} - 1) \sum_{\mathrm{l}=1}^{45} \frac{r_{\mathrm{l}}}{vk} + \sum_{\mathrm{l}=1}^{\mathrm{j}} \frac{r_{\mathrm{l}}}{vk}. \qquad (3)$$

$$a_{\mathrm{j}} = \frac{k_2{}^{\mathrm{j}-1} r_1}{v(k_1 + k_2)^{\mathrm{j}}}, \quad \mathrm{j} = 1, 2, 3\ldots \qquad (4)$$

Page 167:

$$a_{\mathrm{j}}(t) = A_{\mathrm{j}} t^{\mathrm{j}-1} e^{-kt} \qquad (5)$$

$$a_{\mathrm{j}}(t) = \frac{k^{\mathrm{j}-1}}{(\mathrm{j} - 1)!} A_0 t^{\mathrm{j}-1} e^{-kt} \qquad (6)$$

Page 168:

$$d_{\mathrm{i}} = d_0 e^{-\mathrm{li}}, \quad \mathrm{i} = 1, 2\ldots \qquad (7)$$

Vol. 159, No. 3 **December 1980**

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

Reference: *Biol. Bull.,* **159**: 505–560. (December, 1980)

CELLULAR ANALYSIS OF A GASTROPOD (*HERMISSENDA CRASSICORNIS*) MODEL OF ASSOCIATIVE LEARNING

DANIEL L. ALKON

Section on Neural Systems, Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Abbreviations: Excitatory post-synaptic potential, EPSP; inhibitory post-synaptic potential, IPSP; long-lasting depolarization, LLD; tetramethyl ammonium, TMA; ethyleneglycol tetraacetic acid, EGTA; artificial sea water, ASW; cerebropleural ganglion, CPG; resting potential, R.P.

ABSTRACT

Intuitive formulations concerning the nature of cellular substrates for associative learning are considered. Invertebrate models of vertebrate learning are discussed. A cellular analysis of a long-term associative behavioral change is reviewed for the nudibranch mollusc *Hermissenda crassicornis.* Convergence of neuronal pathways which mediate natural sensory stimuli used for associative training have been determined. Primary biophysical changes within single identified neurons are analyzed for their causal role in learning behavior. A sequence of neural processes is proposed to underlie acquisition and retention of an associative behavioral change of *Hermissenda.* Paired sensory stimuli enhance voltage-dependent currents by means of converging sensory pathways. Repeated pairings cause progressive membrane depolarization, which in turn causes long-lasting conductance changes. Biochemical correlates of these phenomena are presented.

INTRODUCTION

The defining features of classical conditioning, which Pavlov produced in his experiments on dogs (Pavlov, 1927), have also been observed in other examples of associative learning. These features (Miller, 1967; Gormezano and Moore, 1969) include (1) the necessity for temporal association of distinct sensory stimuli, (2) improvement of the learned behavior with practice, also known as acquisition, (3) long duration, and (4) stimulus specificity, *i.e.* responses to the conditioned stimulus, but not to other stimuli, are modified.

Copyright © 1980, by the Marine Biological Laboratory
Library of Congress Card No. A38–518
(ISSN **0006–3185**)

Although we can readily conceive of human emotional responses being conditioned by associated trauma, how do we conceptualize the nature of other human learning, far more extensive, but less emotionally charged, in our experience? As humans we learn to remember and recognize another person as an infinitely rich perceptual experience evoked by a name: a visual space or matrix filled with specific ordering of colored points, an auditory space filled with ordering of sound waves by amplitude and frequency, and an emotional space composed of a subtle balance of feelings. What meaning does the simple conditioning of a dog have for these memories of which human consciousness is constructed? Even less obvious is the relevance of behavioral changes of gastropod molluscs. What can a snail be for other snails except a source of a few specific stimuli which elicit stereotypic aggressive, escape, or copulatory behavioral patterns?

According to a reductionist's approach to human learning, implicit in Pavlov's experiments, complex memories might be considered as generated from a host of elemental memories or associations. A view contrary to the reductionist's would hold that human learning is so unique that hosts of elementary associations are not sufficient explanation. Neither view is clearly favored by our current knowledge of animal nervous systems. Although there are many characteristic differences between neural elements of humans as compared to those of other species, none of these differences themselves present obvious advantages for long-term information storage which could be important for learning. This lack of apparent functional uniqueness of human neural cells for long-term change allows at least for the possibility that elemental associative learning processes can be accomplished by elementary neural systems, and that multiplication of these neural systems in higher species provides for multiplication, and thereby increased complexity, of associations rather than for an entirely new or unique learning process.

With the advent of electrophysiologic recording techniques, vertebrate studies have allowed us to conclude that neuronal impulse activity in certain cortical and subcortical structures changes specifically as a result of training procedures. Most of these studies were conducted with electrodes outside of neurons (John and Killam, 1959; Adey *et al.,* 1960; Jasper *et al.,* 1960; Doty, 1961; Buchwald *et al.,* 1966; Livingston, 1966; John, 1967; Cohen, 1969; Olds and Hiramo, 1969; Olds *et al.,* 1972; Evarts, 1973; John *et al.,* 1973; Cohen and Macdonald, 1976; Norman *et al.,* 1977; Berger and Thompson, 1978a, b; Toledo-Morrell *et al.,* 1979). These studies monitored for the most part impulse frequency changes as a function of training. More recently, Woody and his colleagues have utilized intracellular electrodes to measure changes of excitability as a function of a conditioning procedure (Woody and Engel, 1972; Woody and Yaronsky, 1972; Woody and Black-Cleworth, 1973; Woody *et al.,* 1976). What these studies have not revealed are the primary cellular and subcellular changes which cause learned behavior. For example, do the primary changes occur at synapses, axonal branch points, or neural cell bodies? Are there long-lasting biochemical modifications which control membrane conductances? Are there morphological changes? Do neurons grow new processes?

To determine primary neural mechanisms for learned behavior we are limited by existing technology to using a behavioral model in an animal where precise determination of networks is possible. Among so-called "simple-system" preparations which have been investigated as potential models of learning are the crayfish (Bullock and Quarton, 1966; Krasne and Woodsmall, 1969; Zucker *et al.,* 1971; Zucker, 1972a, b), the locust (Horridge, 1959; 1962a, b; Hoyle, 1964, 1965;

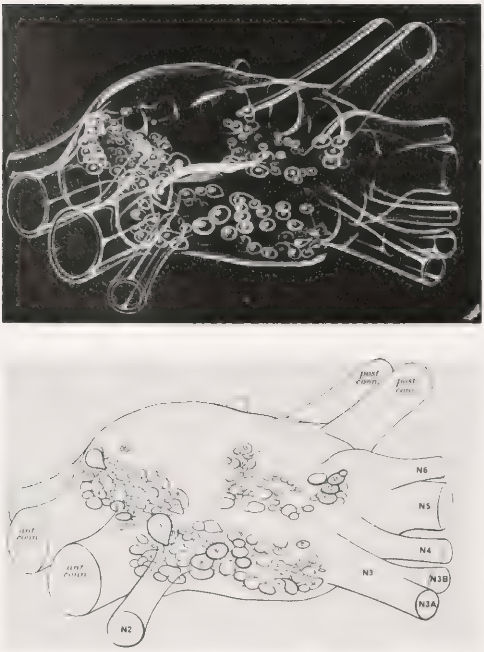


FIGURE 1. A three-dimensional representation (upper sketch) of the metathoracic ganglion of a young adult male cockroach. Note that all the cells except those associated with nerve 2 send their axons out from the ipsilateral nerve trunk. The lower outline indicates some of the cell bodies and connectives investigated (Cohen and Jacklet, 1967). Note that all the cells except those associated with nerve 2 send their axons out from the ipsilateral nerve trunk.

Horridge, 1965; Runion and Usherwood, 1968; Burrows, 1973; Burrows and Hoyle, 1973; Hoyle and Burrows, 1973a, b; Burrows and Horridge, 1974; Pearson and Goodman, 1979), and the cockroach (Eisenstein and Cohen, 1965; Wilson, 1965, 1966; Cohen and Jacklet, 1967; Aranda and Luco, 1969; Disterhoft *et al.*, 1968; Disterhoft *et al.,* 1971; Pearson, 1972). Thus far the nervous systems of these preparations (Fig. 1) have proven rather complex for a comprehensive neural network analysis. To achieve such an analysis, other investigators turned to gastropod molluscs for learning models. A non-associative behavioral change, habituation, was produced with gastropods many years ago (Humphrey, 1930; Harris, 1943; Thorpe, 1963). Hughes and Tauc (1963) produced a decreased amplitude of compound excitatory post-synaptic potentials (EPSPs) in an *Aplysia* neuron by repeated mechanical stimulation applied to the animal's skin. Bruner and Tauc produced a similar decrease in the giant cell of the left pleural ganglion by repeated electrical stimulation of afferent fibers (1964). Subsequently, Kandel, Peretz, Tauc, and their colleagues established more clearly the relationship of decreases in unitary EPSPs to habituation of *Aplysia* behavior (Bruner and Tauc, 1964; Kandel and Tauc, 1964; Bruner and Tauc, 1965, 1966a; Kupferman *et al.*, 1970; Peretz, 1970; Carew *et al.,* 1972; Carew and Kandel, 1973; Eisenstein and Peretz, 1973; Peretz and Howieson, 1973; Peretz and Moller, 1974). These and other workers also analyzed behavioral and cellular aspects of dishabituation, a non-associative stimulus-evoked restoration of a previously habituated response (Kandel and Tauc, 1964; 1965a, b; Tauc and Epstein, 1967; von Baumgarten and Djahanparwar, 1967; von Baumgarten and Hukuhara, 1969; von Baumgarten, 1970; Epstein and Tauc, 1970; Peretz, 1970; Castellucci *et al.,* 1970; Carew *et al.,* 1971). Strumwasser, Jacklet, and others have used *Aplysia* to study neuronal mechanisms of circadian rhythms of behavior (Strumwasser, 1965; Strumwasser *et al.,* 1969; Jacklet, 1977).

During the last 10 years other investigators have turned to a number of gastropod molluscs for examples of associative learning behavior mediated by analyzable neural pathways. With the exception of the nudibranch *Hermissenda crassicornis* (Fig. 2; Alkon, 1974a, b) these molluscs experience chemical (and/or touch) stimulation of the tentacle or oral veil during the training paradigm.

In considering possible preparations it seemed reasonable to me that to understand quantitatively how a nervous system causes associative learning behavior, it would be advantageous to use natural stimuli transduced by sensory receptors during an organism's normal life cycle. The effects of these stimuli might then be followed step by step from the receptors to interneurons and ultimately to motor neurons and muscular activity. Gastropods were attractive because of their relatively small number of neurons yet fairly elaborate behavioral capability. It also seemed reasonable that with such a small number of neurons, the animal was more likely to be capable of intermodal than of intramodal sensory associations. Natural stimuli for the training regimen seemed important to me for another reason: the patterns of natural stimuli encountered by the animal in its environment should determine the range of possible learned behaviors adaptive within a given biological

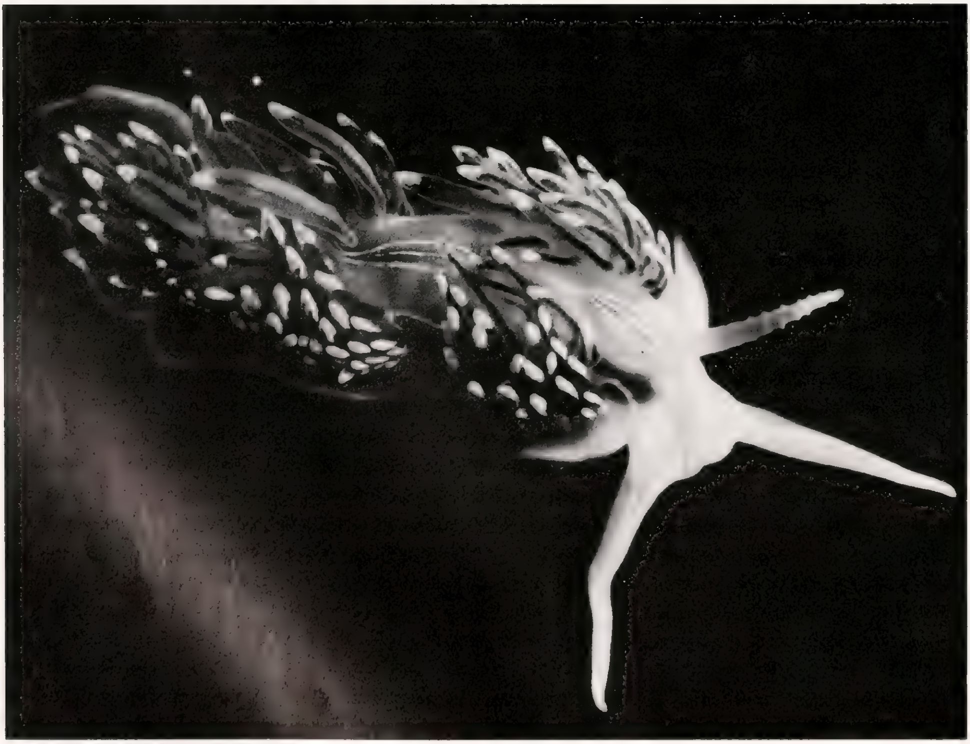


FIGURE 2. The nudibranch mollusc *Hermissenda crassicornis.* Note the small black spot (the right eye) at the base of the lower rhinophore. Length of the animal is ~ 4 cm.

niche. Brief dissection of a transparent dorsal integument reveals the circumesophageal nervous system of *Hermissenda*, including the receptor organs (Fig. 3A) of three distinct sensory pathways: visual, statocyst, and chemosensory. The easy accessibility of the eyes, statocysts, and central ganglia to electrophysiologic recording techniques, and the close proximity of the eyes to the statocysts, encouraged consideration of *Hermissenda* as a model system.

During the light period of a 6.5 hr light: dark cycle, light elicits a positive behavioral response from *Hermissenda* (Fig. 4A). The animal increases its general level of motor activity and moves toward a light source (Alkon, 1974; Lederhendler *et al.,* 1980). In response to rotation the animal moves toward the center of rotation, thereby reducing the centrifugal force it experiences. Similarly, the animal tends to move up a vertical gradient, *i.e.,* away from the direction of the earth's gravitational force (Alkon, 1973, 1976). In response to vigorous rotation the animal also contracts the musculature of its body wall and "clings" to surfaces (Fig. 4B). This "clinging" minimizes the turbulence the animal would experience were it free of any surfaces. In all then, rotation can be regarded as a negative or aversive stimulus with effects the animal reduces by its responses.

When light that elicits a positive response is repeatedly paired with rotation, an aversive stimulus, the animal's subsequent reaction to light is markedly less positive (Alkon, 1974), *i.e.,* the animal no longer approaches a light source (Fig. 5). Crow and Alkon (1978) showed that this behavioral change is truly associative (that is, randomized light and rotation did not produce the same effect), persists for at least several days after training, and increases with practice (Fig. 6A, B). Specificity of this training effect was suggested by the fact that trained animals did not show changes in response to food. Specificity was further indicated by the observation that animal's response to light but not darkness is modified by stimulus pairing (Crow and Offenbach, 1979; Farley and Alkon, in preparation). Stimulus pairing also did not modify the animals' negative geotactic responses as demonstrated with movement on a vertical gradient (Farley and Alkon, in preparation). Thus, this behavioral change of *Hermissenda* shows defining features of, and can serve as a model for, associative learning as described for vertebrate species.

What is the possible physiologic significance of this associative behavioral change? *Hermissenda,* like other intertidal molluscs, can be seen to approach the water's surface during daylight hours and is found at lower depths at night (Harrigan and Lederhendler, in preparation). This behavior can be explained at least in part by the animal's positive phototaxis during the day, as demonstrated by Lederhendler *et al.* (1980), and negative phototaxis at night. This cyclic movement has adaptive value in that hydroids, microorganisms on which *Hermissenda* feeds, are concentrated in illuminated areas.

During periods of increased oceanic turbulence, such as might occur in a storm, any *Hermissenda* approaching the water's surface during daylight would be shaken vigorously—*i.e.,* they would be shaken in association with light. This shaking was approximated by the rotation stimulus conditions originally paired (Alkon, 1974) with light. *Hermissenda,* then, as it approached the water's surface would experience more light in association with turbulence. It is possible that repetition of this association in the field, like the association of light and rotation in the laboratory, would result in reduced movement of the animals toward the water's surface, thereby minimizing the turbulence subsequently experienced. Persistence of re-

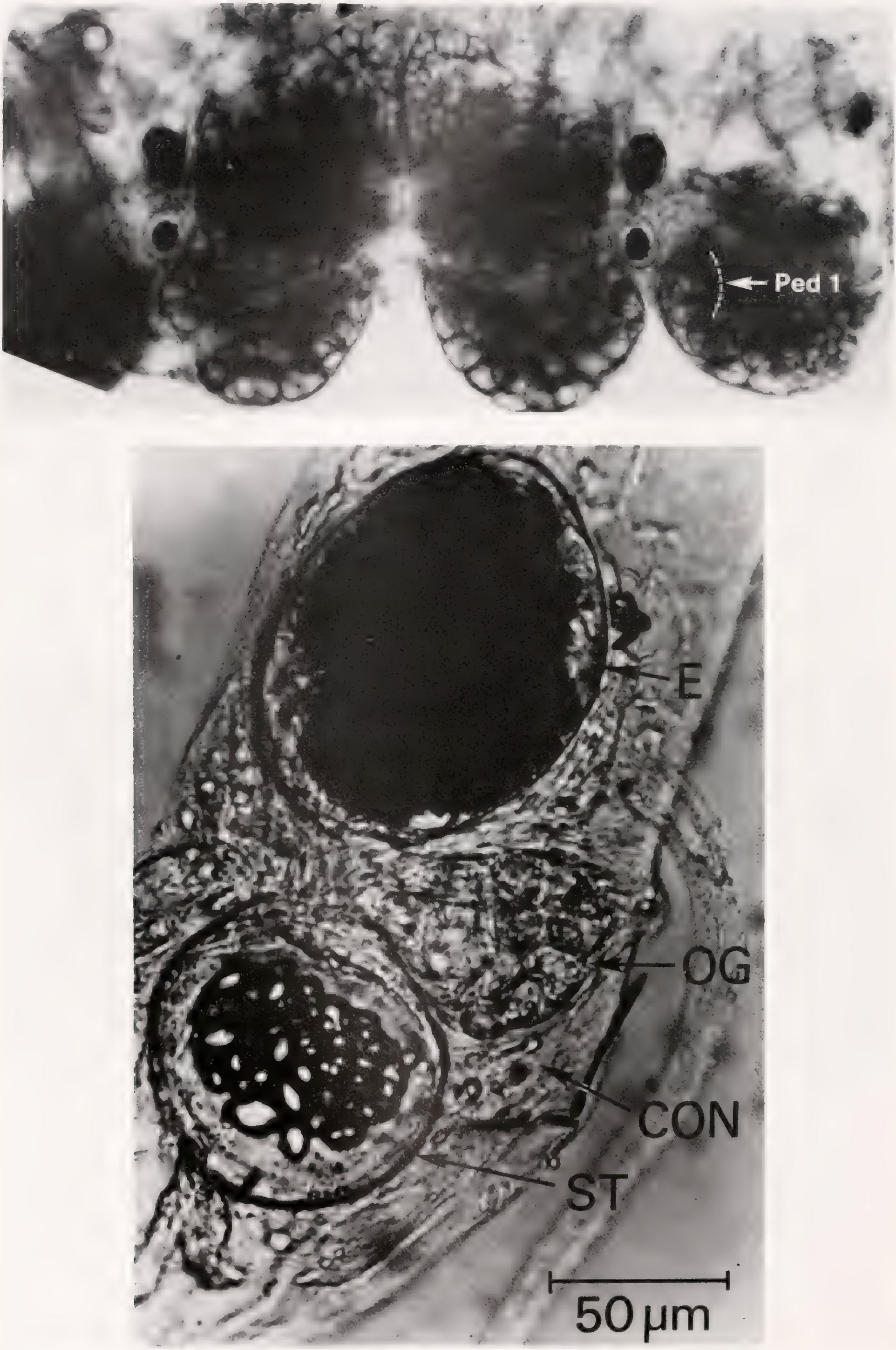
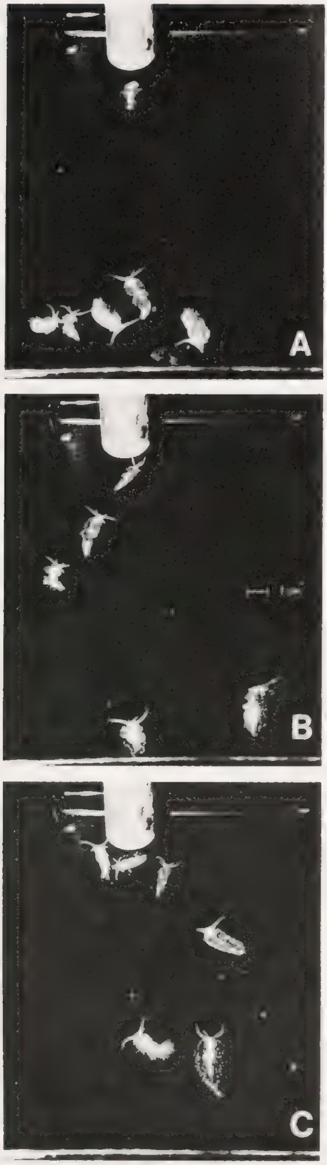


FIGURE 3A. (Top) *Hermissenda* circumesophageal nervous system. The photomicrograph is of a living preparation. The statocysts lie between the two central cerebroplural



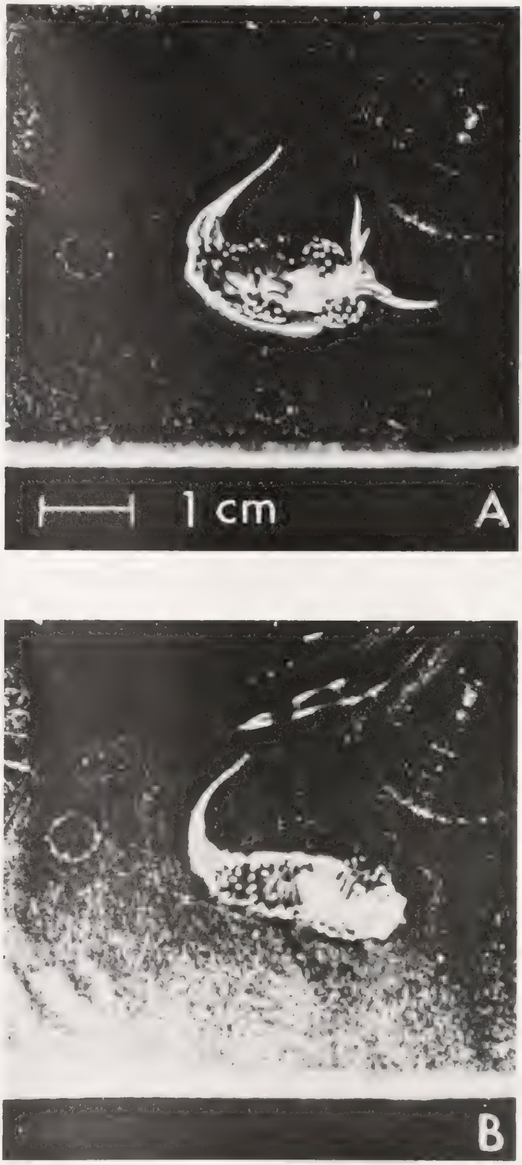


FIGURE 4A. (Left) Response of animals in experimental chamber to test light spot. A, B, C animals move toward and into light spot within 20 min. Spot (indicated by dashed lines) is obscured by stroboscopic flash in B, C. Calibration: 1 cm (Alkon, 1974).

FIGURE 4B. (Right) Response of *Hermissenda* to rotation. A, immediately before and B, immediately after 20 sec of rotation at 200 rpm. Immediately after and during rotation the entire animal is contracted (Alkon, 1974).

---

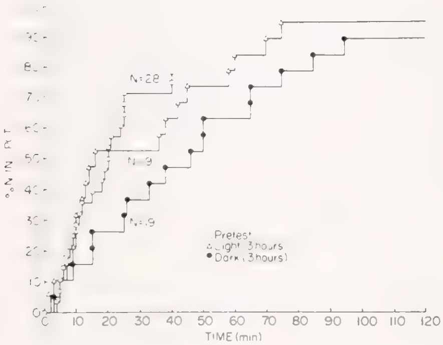
ganglia and the two lateral pedal ganglia. Immediately above the statocysts are the optic ganglia (transparent) and above them are the eyes (darkly pigmented). Ped. 1 indicates a putative motor neuron.

FIGURE 3B. (Bottom) Combined (isolated) sensory structures. E, eye; OG, optic ganglion; ST, statocyst; and CON, capsule of connective tissue (Heldman *et al.*, 1979).

duced positive phototactic behavior over several days of a storm, then, could on balance have survival value.

For *Pleurobranchaea* (Fig. 7A), another gastropod used as a learning model, a food substance touched the tentacle, paired (or unpaired) with electric shock to the same tentacle and/or the head region (Mpitsos and Collins, 1975). A behavioral change was measured when the animals withdrew on subsequent touching with the food substance instead of the pre-training "bite and strike" feeding behavior. This behavioral change was assessed by repeated touching of the tentacle until withdrawal or feeding was elicited. More animals withdrew from the food following pairing with electric shock than following an unpaired training regimen. Gelperin found that the terrestrial slug *Limax maximus* (Fig. 7B) will avoid food substances after paired presentations with CO_2 poisoning (Gelperin, 1975). Within the last year, Walters *et al.* (1979) paired chemical stimulus presentations with electric shocks to the head region of *Aplysia californica*. *Aplysia* responses to test shocks across the tail were modified by paired but not by unpaired stimulus presentations.

These unpaired stimulus presentations control only to a limited extent for nonassociative behavioral changes of *Pleurobranchaea* and *Aplysia*. There is a fixed temporal relationship or "association" of stimuli even with these unpaired paradigms. A more unequivocal control (Rescorla, 1967), in which food presentation has no fixed temporal relationship to shock, would clearly demonstrate the associative nature of this behavioral change. This is not entirely possible for the *Pleurobranchaea, Limax,* or *Aplysia* models, since only a few stimuli are presented over several hours during a training session. Range of variation in the temporal relationships among stimulus presentations adequate to approximate their randomization is therefore not realistic. In a more recent study, stimulus specificity for the be-



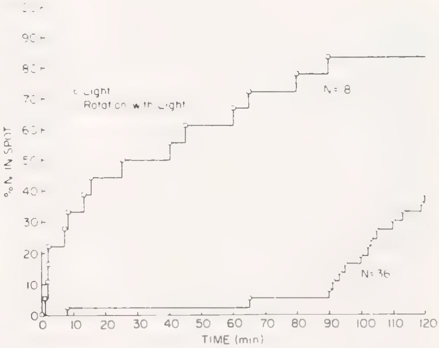


FIGURE 5A. (Left) Kaplan-Meier (1958) curves for percent of animals which have reached a test light spot as a function of time. Squares: Animals tested after 10 min of darkness in experimental chamber. Triangles: Animals tested after 3 hr of 70-sec light intervals (see Methods). Circles: Animals tested after 3 hr of complete darkness. These data were obtained from photographic records. Animals were not removed when they reached the test spot nor was interaction between animals prevented. By the Gehan-Breslow test procedure the three groups are not significantly different (Alkon, 1974).

FIGURE 5B. (Right) Kaplan-Meier (1958) curves for percent of animals which have reached a test light spot as a function of time. These data were obtained by direct observation of animals entering the test light spot. Animals were removed when they reached the test spot and interaction between animals was prevented. Squares: Animals tested after 3 hr of light intervals. Circles: Animals tested after 3 hr of rotation intervals associated with light. The two groups are significantly different ($P < 0.001$) by the Gehan-Breslow test procedure (Alkon, 1974).

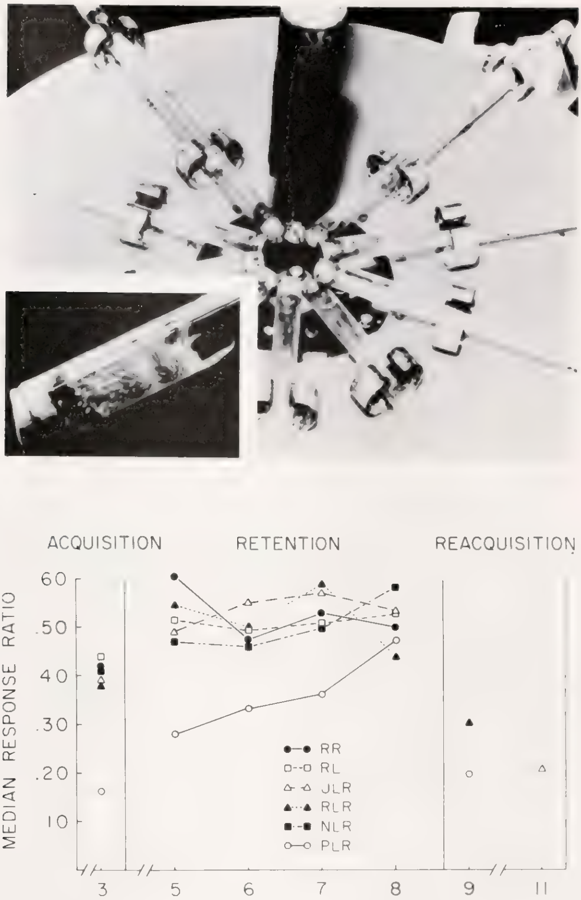
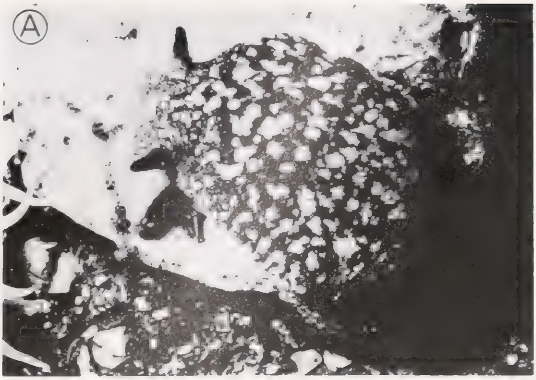


FIGURE 6. (Top) Training and testing apparatus. The response latencies to enter a light spot projected onto the center of the turntable by an overhead illuminator were recorded automatically when the *Hermissenda* moved toward the light source (direction of arrows) and interrupted the light between illuminator and photocells (arrowhead). (Inset) *Hermissenda* was subjected to different behavioral treatments, consisting of light and rotation, while confined to the end of glass tubes filled with seawater. (Bottom) Median response ratios for acquisition, retention, and reacquisition of a long-term behavioral change in response to a light stimulus in *Hermissenda.* Solid circle, random rotation; open square, random light; open triangle, unpaired light and rotation; solid triangle, random light and rotation; solid square, nothing; and open circle, paired light and rotation. The response ratio in the form of 1–A/(A + B) compared the latency during the test (A) with the baseline response latency (B). Group data consist of two independent replications for all control groups and three independent replications for the experimental group (Crow and Alkon, 1978).

havioral change of *Pleurobranchaea* was assessed following pairing of food substances with electric shock; Davis *et al.*, 1980). A clear demonstration of stimulus specificity would add support to the hypothesis that a truly associative behavioral change was involved. Significant differences, however, between testing an avoidance response with squid extract and testing with *Corynactis* extract were not demonstrated following paired presentations of squid and electric shock. In fact, on



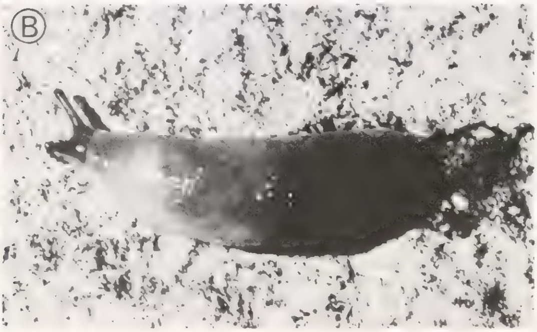


FIGURE 7A. (Top) *Pleurobranchaea californica*: a large notaspidian opisthobranch common to the subtidal waters of the California coast. It has lost its shell completely and bears a gill on the right side of the body under the large mantle cover, which is continuous with the expanded frontal veil. The veil also bears the tentacles (animal size = 15 cm long) (courtesy of Dr. Alan M. Kuzirian).

FIGURE 7B. (Bottom) *Limax maximus*: common terrestrial slug (Pulmonata: Stylommatophora) having secondary reduction and concealment of the shell and a pair of eyes borne on the tips of the tentacles (animal size = 4.5 cm) (courtesy of Dr. Alan M. Kuzirian).

the third day following training the *Corynactis* avoidance response was significantly greater than controls, whereas the squid response was not. Also inconclusive were attempts to demonstrate differential avoidance responses after paired presentation of shock and one food substance together with unpaired presentation of a second food substance. Differential avoidance response behavior was observed on the second but not the first or third days. Some significant differences occurred for a "bite-strike" response. Most apparent, however, for the differential training procedures, was the finding that the animals in general showed more withdrawal behavior than for the single stimulus pairing procedure. This suggests that animals receiving more food stimulus presentations, regardless of their relation to shock, withdraw more. Since the animals were unfed for the entire 2 weeks of training and testing, the food used and ingested during training might significantly influence the animals' relative states of starvation.

In fact, a general difficulty with those gastropod models using chemical (food substance) stimuli is the control of satiation effects. In none of these studies (using food paired with other stimuli) were the animals fed daily or were the effects of inanition monitored. The electric shocks used for the *Pleurobranchaea* and *Aplysia* models and the CO_2 poisoning for *Limax,* when paired with food, might influence the animal's ability to feed and thus its state of satiation. The animal's subsequent response to food might reflect how much it ingested specific food substances or extracts during and/or between stimulus presentations, rather than the learning of an association between a chemical stimulus and shock (*Pleurobranchaea* and *Aplysia*) or food and CO_2 poisoning (*Limax*). States of inanition have recently been found to have important effects on gastropod responses to reproductive (Davis *et al.*, 1974), visual (Alkon *et al.*, 1978), and touch (Lukowiak, 1979) stimuli, and to influence vertebrate neuronal responses to sensory stimuli (Nienhuis and Olds, 1978). A final difficulty with these models just mentioned concerns any possible meaningfulness for the animals in their natural environments. The use of electric shocks of sufficient magnitude to affect a substantial portion of the animal's body, or CO_2 poisoning, which has diffuse toxic effects, obviates comparisons to behavior in response to natural stimuli.

Cellular analyses of associative learning

Associative learning of any species should be explainable by the functions of cells. Theoretically, behavioral changes must result from neural changes and neural changes must depend on biophysical and biochemical processes capable of long-term transformation. Developmental changes of neural systems, such as those observed in cats by Hubel and Wiesel, Hirsh and Spinelli, Blakemore, and others (Wiesel and Hubel, 1965; Pribram *et al.*, 1967; Blakemore *et al.,* 1970; Hubel and Wiesel, 1970; Hirsh and Spinelli, 1971) are too slow to explain rapid, sometimes one-trial, learning. Growth of neural branches over any significant distance (> 1 μm) requires hours if not days. Rapid learning, therefore, seems most reasonably accomplished via pre-existing neural circuits or systems. Again, this is an intuitive formulation which motivates a working hypothesis: genetically specified neural circuits hold the potential of environmental specification by learning from sensory experience.

Investigation of neural changes during learning must begin with the question, what are the genetically specified neural circuits; what are the invariant aspects of gastropod neural systems? The second question is: Given genetically determined limits of variance, what is the variance specific to and causal of learning behavior? We must return, therefore, to what is known of the neural systems in these gastropod preparations: in general, very little. Although there are relatively few neurons, only some are identifiable from preparation to preparation, and many are not large and not superficially located on the ganglia, thereby making intracellular recordings less practical. In addition, considerable variability of neural structure has been demonstrated for gastropod and arthropod preparations (Lubbock, 1858; Burrows, 1973; Macagno *et al.*, 1973; Pitman *et al.*, 1973; Stretton and Kravitz, 1973; Altman and Tyrer, 1977; Goodman, 1978; Pearson and Goodman, 1979; Calabrese, in preparation). Because most synaptic interactions of gastropod neurons occur on axons at considerable distances from the cell bodies impaled by the intracellular electrodes, distortion of synaptic signals is likely and identification of direct or monosynaptic interactions by the necessary combination of electrophysiologic and morphologic criteria has only rarely been achieved.

In fact, in many respects, vertebrate sensory pathways are better understood than those of gastropods. We know, for example beginning with the sensory receptors, successive neuronal groups which constitute the pathways for touch,

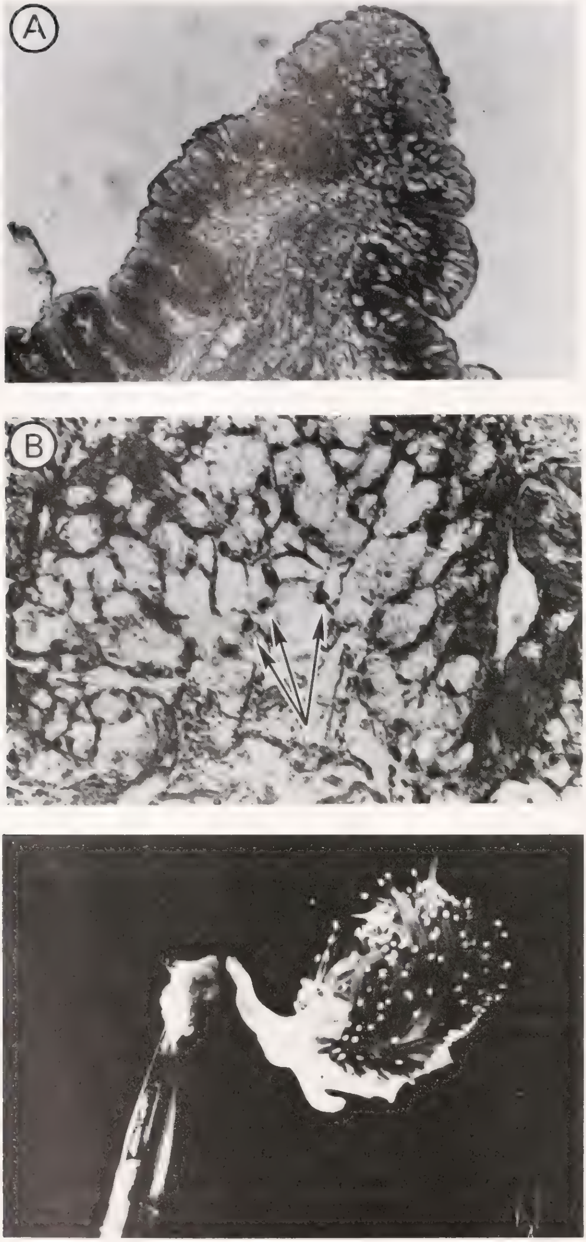


FIGURE 8. Tentacle of *Hermissenda* stained for neural cells (Bodian technique). A. Longitudinal section of tentacle tip. Darkly stained areas represent loci of presumed chemoreceptors (200 ×). B. Higher magnification (1000 ×) of presumed neural network immediately below receptors. Arrows indicate putative individual neurons, ~ 3 μm in diameter. C. Tentacular response of *Hermissenda* to touch of food substance (squid). Animal is ~ 3 cm long.

vibration, visual, auditory, and to some extent olfactory sensation (Shepherd, 1979). For neural systems such as the retina (Dowling and Boycott, 1965; Lasansky, 1971) and cerebellum (Llinas *et al.,* 1972; Llinas and Hess, 1976) synaptic interactions occur on or near cell bodies, and readily recognizable ultrastructural features can be used to demonstrate synaptic loci. Finally, because of the considerable redundance within these vertebrate neural systems, knowledge of neural circuitry for a unitary neural group (*e.g.* receptor-horizontal-bipolar-amacrine-ganglion cell aggregate) can be extended to some degree to a host of other comparable neural groups. Where, however, does chemosensation begin for *Pleurobranchaea, Helix,* or *Aplysia* (Stinnakre and Tauc, 1969; Fredman and Jahan-Parwar, 1980)? Our experience with *Hermissenda* (Agersborg, 1922, 1925; Alkon *et al.,* 1978) suggests the presence of receptors and neural plexi (Fig. 8) on the tentacular structures.

Even after these chemoreceptor loci are unequivocally identified, intracellular recording, if past experience with chemoreceptor recording (Getchell, 1977) is any guide, may be difficult to accomplish. Higher order chemosensory neurons and their synaptic interactions have also yet to be comprehensively studied in gastropods, although this work has begun (Fig. 9; Gillette and Davis, 1977; Spray *et al.,* 1980; Gillette *et al.,* 1980). The neural pathways which mediate the effects of electric shocks used for training gastropods are even less well understood. Considering the preliminary nature of neural network analysis of chemoreception and sensation of electric shocks, it is not surprising that no primary neural correlate specific to training with these stimuli in a paired paradigm has been clearly demonstrated.

Determining the primary neural changes specific to pairing might be especially difficult if, as has been suggested, neural networks with a large number of small neurons are situated on the tentacles themselves. It is for this primary neural change specific to pairing, however, that gastropods are particularly needed. As already mentioned, a number of workers have recorded neural changes following and accompanying associative learning by vertebrates (Fig. 10; Berger and Thompson, 1978a–c). The question that these workers may not be able to address with present technology is: What is the causal sequence, beginning with the training stimuli, that ultimately results in the behavioral change? Recording, from gastropod command or motor neurons, neural changes specific to pairing of chemosensory and shock stimuli, will not substantially enhance our knowledge of mechanisms of associative learning if these changes are simply a consequence of changes in synaptic input from other neurons that are themselves the site of primary or causal neural modification responsible for the learning behavior.

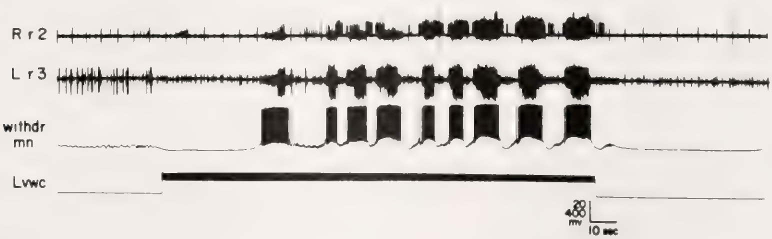


FIGURE 9. Intracellular stimulation of the left ventral white cell (Lvwc) drives the feeding rhythm, recorded intracellularly from a withdrawal motor neuron (withdr mn) and extracellularly from roots 2 and 3 of the buccal ganglion (Rr2, Lr3) (Gillette *et al.,* 1980).

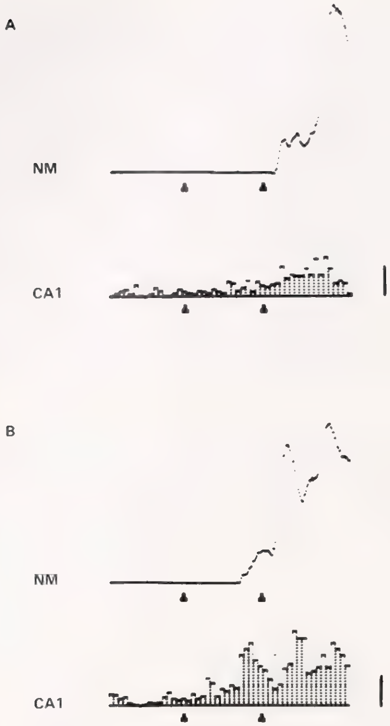
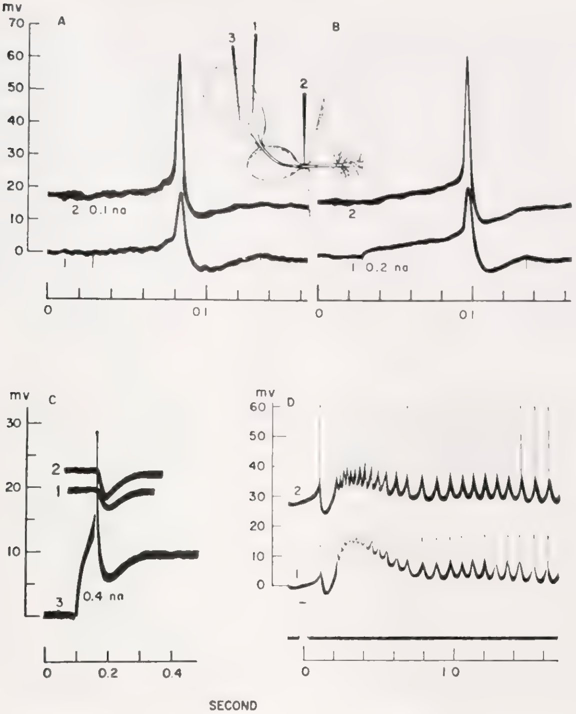


Figure 10. Hippocampal multiple unit response recorded during NM classical conditioning. Upper trace: average NM response for one block of eight paired trials. Lower trace: hippocampal multiple unit poststimulus histogram (15-msec time bins) for same block of eight paired trials. First cursor indicates tone onset; second cursor indicates air puff onset. Total trace length equals 750 msec here and in all subsequent histograms. (A) Second block of conditioning training, Day 1. (B) Tenth block of conditioning training, Day 2. Vertical calibration in A and B equals 25 unit counts per 15-msec time bin (Berger and Thompson, 1978).

Neural Organization in *Hermissenda*

To find primary neuronal changes responsible for learning, a comprehensive study of *Hermissenda* pathways was undertaken, beginning with intracellular recording from and histologic identification of photoreceptors in the eye and the hair cells in the statocyst, a primitive vestibular organ. Investigations then focused on second order neurons, other interneurons and, more recently, putative motorneurons. Sites of convergence between the visual and statocyst pathways and later the chemosensory pathway were also determined (Alkon and Fuortes, 1972; Alkon and Bak, 1973; Alkon, 1973a, b; Detwiler and Alkon, 1973; Alkon, 1974, 1975, 1976a, b; Alkon *et al.*, 1978; Alkon and Grossman, 1978; Akaike and Alkon, 1980).

In general the interactions encountered between neural elements within these sensory pathways included both excitatory and inhibitory chemical synapses and electrical synapses. One example of an apparent non-synaptic interaction (Alkon and Grossman, 1978) was also found. Many of the chemical synaptic interactions appeared to be monosynaptic. In these cases discrete synaptic potentials followed with a brief latency, in a one-for-one fashion, impulses of the presynaptic cell (Figs. 11, 12). However, it is not unequivocally established that these are monosynaptic interactions.



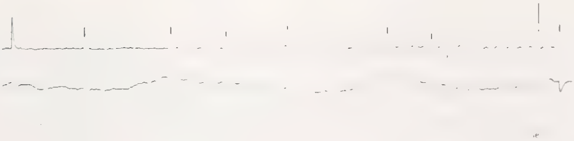


FIGURE 11. (Above and middle) Responses recorded from soma and axon of same cell. The distance between the two electrodes was 80–100 μm. A) Responses to a step of depolarizing current through axonal electrode. B) Response to a step of current through the soma electrode. In either case the spike is larger and earlier in the axon. C) Two electrodes were inserted, respectively, in the soma and axon of one receptor and a third electrode was inserted in the soma of a second receptor. A step of depolarizing current through this third electrode evokes a spike in one receptor and a hyperpolarizing synaptic potential in the other. The synaptic potential is larger and has shorter time to peak in the axon. D) Responses to a flash of light. The generator potential is larger at the soma (*lower trace*) while the spikes are larger at the axon. Down-going bars in (A) and (B) indicate timing of current steps (Alkon and Fuortes, 1972).

FIGURE 12. (Below) Simultaneously evoked EPSPs in a second order (cerebropleural ganglion) visual neuron and IPSPs recorded from an ipsilateral Type A photoreceptor. The second order neuron was hyperpolarized by injecting–1.0 nA D.C. Current. Voltage calibration: 20 mV. Arrows indicate IPSPs in the Type A cell simultaneous with small EPSPs in the second order visual neuron (Akaike and Alkon, 1980).

In our investigation of *Hermissenda's* neural systems we used mainly electrophysiologic criteria (obtained by simultaneous intracellular recording from pre- and post-synaptic elements) to characterize neural interactions. Ultimately, however, morphologic criteria (Figs. 13, 14) must be relied on for more complete description of these interactions. Our observations, although incomplete, directed our efforts within these neural systems to a causal sequence of changes likely to be

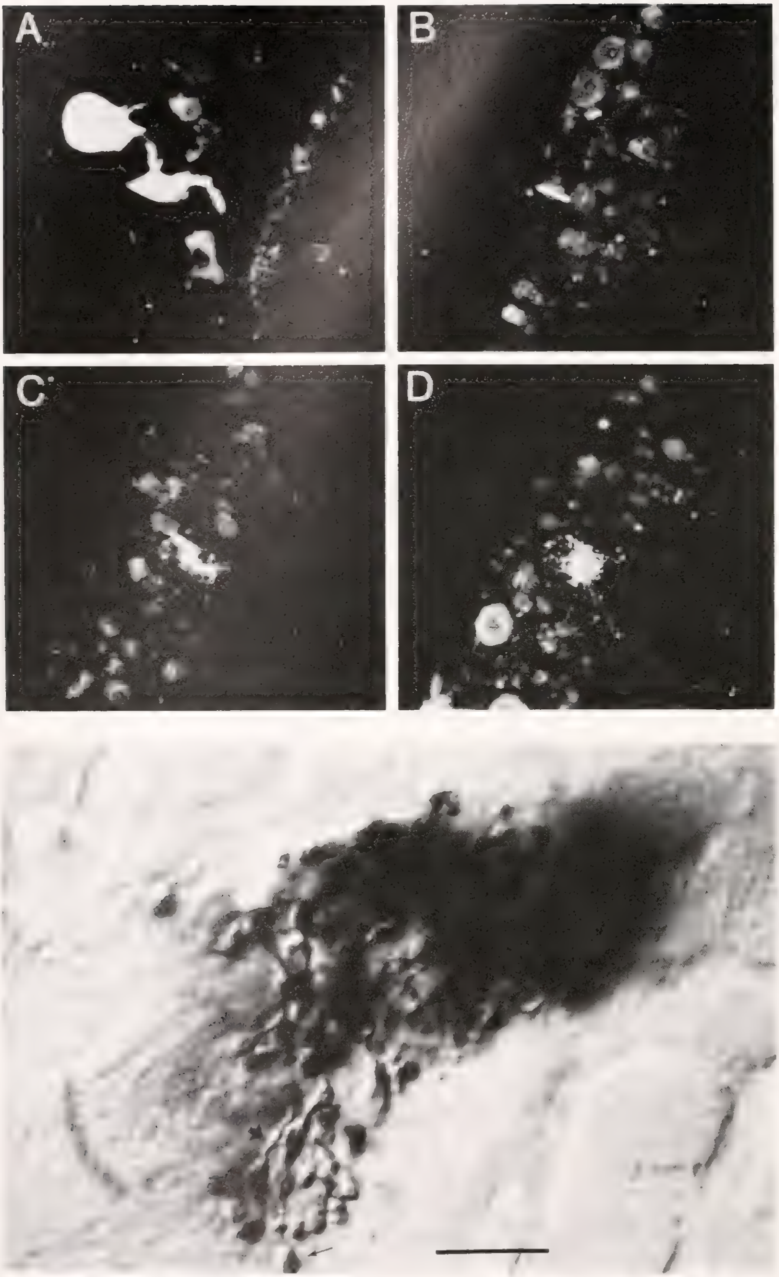


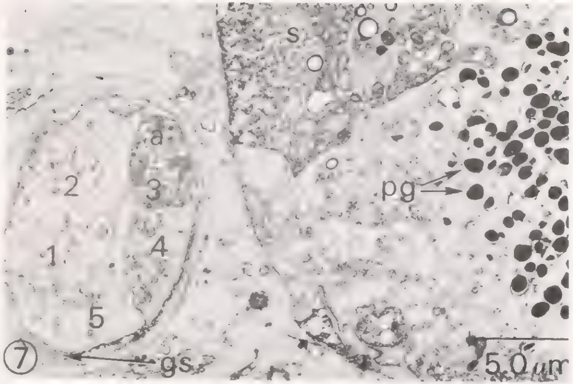
FIGURE 13. Type B photoreceptor stained by iontophoretic injection of Procion yellow. Dark field photomicrographs demonstrate the fluorescent dye within the cell and its processes. A) cross section of cell soma and axon. B–D) details of axon and terminal branches. 480 × (Alkon, 1976). E) Nomarski optical section of HRP-labeled terminals of a photoreceptor in *Hermissenda crassicornis.* × 2400. Scale bar = 10 microns; thick arrow = connecting process; thin arrow = terminal swelling (courtesy of S. Senft).

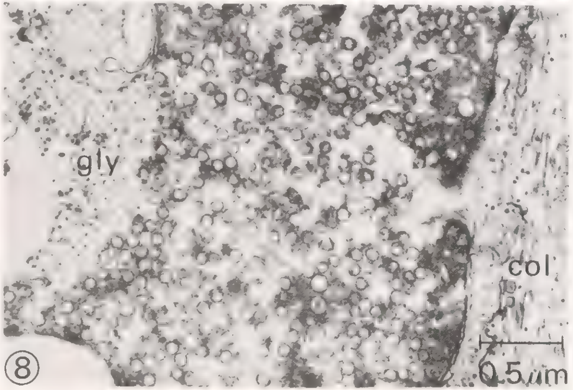
responsible for behavioral changes acquired during the associative learning paradigm. This was possible only after we had substantial confidence in the reproducibility from animal to animal of the observed interactions. For this it was necessary to study repeatedly, in many animals, the same interactions, as will now be briefly described.

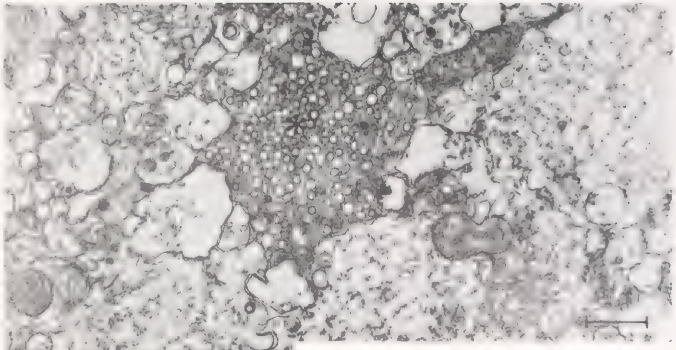
Photoreceptors

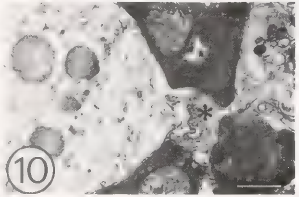
Each of the two *Hermissenda* eyes contains five photoreceptors. Two of these, the Type A photoreceptors, are located in the ventral anterior part of the eye and the other three in the dorsal posterior region. Type A photoreceptors have spikes of 45 mV (based on intracellular recordings from > 150 cells), are silent in darkness, are less sensitive to light than Type B cells, and have little or no interaction with each other. (Of Type A cell pairs within the same eye from 12 different animals, only one type A cell showed any response, a slight hyperpolarization, to the other Type A's firing at 20 Hz, elicited by a positive current pulse). In darkness, Type B cells, with spikes of 15 mV average amplitude (based on intracellular recordings from > 400 cells) develop depolarizing waves (3–8 mV) at infrequent and irregular intervals (0.5–30 sec). At least some of these waves are excitatory post-synaptic potentials (EPSPs) which arise from the ipsilateral optic ganglion (Tabata and Alkon, 1979). Type B photoreceptors are all mutually inhibitory (> 100 pairs in different preparations). In an early study (Alkon and Fuortes, 1972) of synaptic interactions between Type A and Type B photoreceptors (50 pairs), the following relations were found: reciprocal inhibition in 22 pairs; inhibition of A upon B in 10 pairs; inhibition of B upon A in 12 pairs, and no interactions in 6 pairs. Subsequent recording experience from A-B pairs has indicated that it is the lateral Type A photoreceptor which has reciprocal inhibitory interactions with the Type B cells in the same eye. Simultaneous intracellular recordings from three or more photoreceptors in the same eye indicated that there are, in each eye, two Type A and three Type B photoreceptors. This is consistent with an earlier histologic study (Fig. 15; Stensaas *et al.*, 1969), which showed five axons exiting from the eye. The location of the microelectrode impaling class A cells suggests that they correspond to cells I and II, classified by histologic criteria (Stensaas *et al.*, 1969) and that the class B cells correspond to cells III through V (Fig. 15). All five photoreceptors depolarize in response to flashes of light. With very dim flashes at least one and probably two Type B photoreceptors depolarize and the two Type A photoreceptors hyperpolarize.

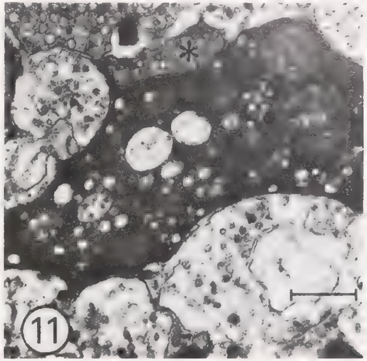
A number of experiments indicated that the locus of the synaptic interactions between photoreceptors (as well as between photoreceptors and other neural elements described below) is at their terminal branchings (Fig. 16) within the pleural ganglia. Iontophoresis of the fluorescent dye Procion Yellow into the photoreceptor somata within the eye reveals an axon which passes through the optic ganglion (Fig. 13) without branching and ends in a spray of fine branches immediately after entering the pleural ganglion. A high resolution Nomarski technique for visualizing these endings (Senft *et al.*, in preparation) reveals detailed structures of these branches and their endings (Fig. 13E). Severing the optic nerve immediately before it enters the pleural ganglia abolishes the inhibitory interactions while preserving the response to light and sometimes the spikes. Finally, recording simultaneously in the axon (at its point of entry into the pleural ganglion) and soma of a photoreceptor shows a decrement of the inhibitory potential (elicited by impulses in another impaled photoreceptor) which is never less than the decrement of hyperpolarizing potentials produced by currents in the axon electrode (Fig. 11). This last experiment indicated that the inhibitory post-synaptic potentials (IPSPs) cannot arise proximal to the axonal recording site. A more recent investigation involved injection of horseradish peroxidase into two Type B


7
s
pg
5.0 µm
a
3
4
2
1
5
gs


8
gly
col
0.5 µm


9
*


10
*


11
*

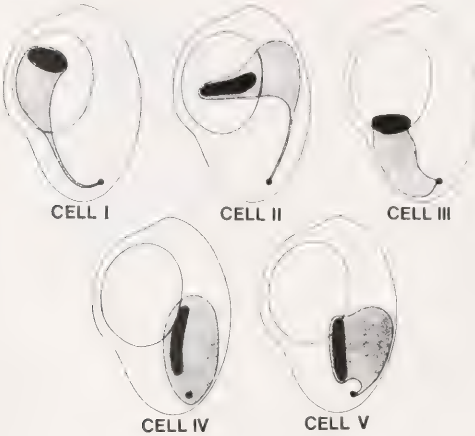


FIGURE 15. Schematic representation of *Hermissenda* eye showing the general configuration of the five photoreceptors (dark stipple), the rhabdomere (black) beneath the lens, and the course of axons which enter the optic nerve (black dot). Cell IV is situated above cell V, and their rhabdomeres are apposed (Stensaas *et al.*, 1969).

photoreceptors within the same eye. Electron microscopic sections revealed a faint and a darkly stained cell. The axons of these two cells could then be traced by thick and thin sections to sites of apposition between faint and darkly stained endings ~ 30 μm from the optic nerves' entry point into the pleural ganglion (Fig. 14). These experiments, taken together, provide strong evidence that the photoreceptors synapse on each other at their terminal branchs within the pleural ganglion.

Optic ganglion cells

There are 14 cells in each optic ganglion (Stensaas *et al.*, 1969). The axons of these cells join those of the photoreceptors to form the optic nerve (10–20 μm), which enters the lateral aspect of the pleural ganglion (Fig. 16). The axons of optic ganglion cells continue to course in a well-defined tract approximately 300 μm into the pleural ganglion. Optic ganglion cells (except for one, the largest)

FIGURE 14A. (Numbered 7) Electron micrograph showing HRP-labeled photoreceptor soma (s) and cross-section of optic nerve. The pigment cells contain numerous pigment granules (pg). The optic nerve consists of five axons (1–5) surrounded by a glial sheath (gs). A darkly labeled axon (a) corresponds to the labeled soma. A partially filled area indicated by arrow (bottom center) may be the result of some leakage from a previous unstable electrode penetration (Crow *et al.*, 1979). A faintly stained axon, apparent in the optic nerve, was stained during this previous penetration.

FIGURE 14B. (Numbered 8) Electron micrograph of an axon hillock of an HRP-labeled photoreceptor. The area contains numerous vesicles 60–80 nm in diameter, and granular material thought to be glycogen (gly). Outside the stained axon are collagen fibrils (col). A small process on the right of the micrograph invaginates the axon (Crow *et al.*, 1979).

FIGURE 14C. (Numbered 9) Electron micrograph of a terminal process following injection of a single Type B photoreceptor. The terminal process (gray area, upper center, with asterisk) contains numerous clear round vesicles and abuts several clear processes. Scale bar: 0.5 μm (Crow *et al.*, 1979).

FIGURE 14D. (Numbered 10, 11) A moderately labeled terminal process (asterisk, upper center) surrounded by three darkly labeled processes following injection of HRP into two Type B photoreceptors. Scale bar: 0.5 μm (Crow *et al.*, 1979).

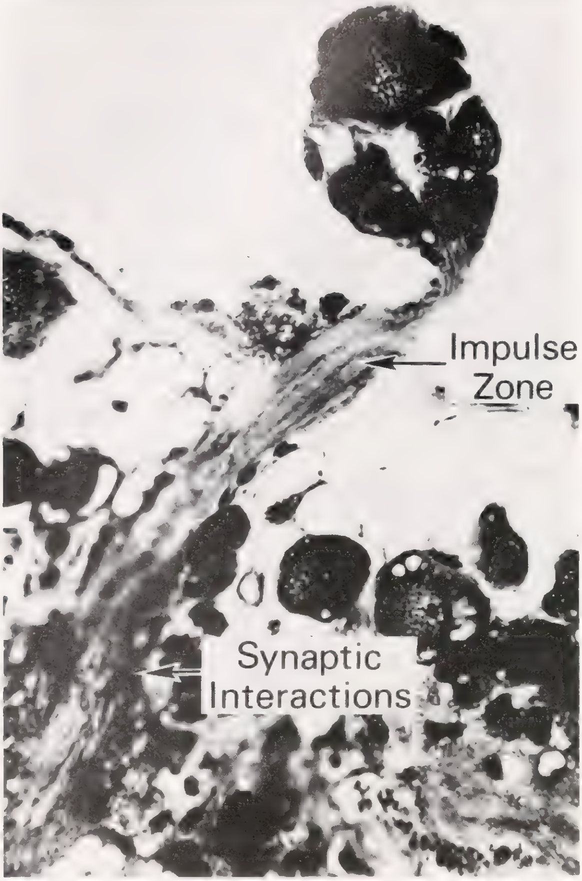


FIGURE 16. 25 μm thick section stained with toluidine blue showing the optic ganglion (40 μm across) and "optic tract," containing 19 axons: of 5 photoreceptors and 14 optic ganglion cells. The largest cell is unresponsive to light. "Impulse zone" indicates excitable focus which generates both optic ganglion and photoreceptor impulses. "Synaptic interactions" indicates area of terminal branches of photoreceptors (Alkon, 1973).

are spontaneously active in darkness and hyperpolarize with a transient cessation of activity in response to a light flash (Fig. 17).

Type B (demonstrated with > 100 pairs) but not Type A photoreceptors (demonstrated with 30 pairs) inhibit ipsilateral optic ganglion cells. This synaptic inhibition produced the hyperpolarizing response of optic ganglion cells in response to light (Fig. 17). Present evidence suggests that the Type B photoreceptor inhibits the optic ganglion cell monosynaptically (Alkon, 1973). A subsequent analysis of photoreceptor-optic ganglion cell interactions has revealed many additional details of Type B photoreceptor interactions with optic ganglion cells (Tabata and Alkon, 1979; Tabata and Alkon, in preparation). Of particular interest is a synaptic excitation of the Type B cells by at least one ipsilateral optic ganglion cell. The site of these EPSPs probably occurs within or near the optic ganglion itself (*i.e.*, nearer than the site of IPSPs between photoreceptors) since they can often be transiently observed after the optic nerve has been severed near its point of entry into the pleural ganglion (Tabata and Alkon, in preparation).

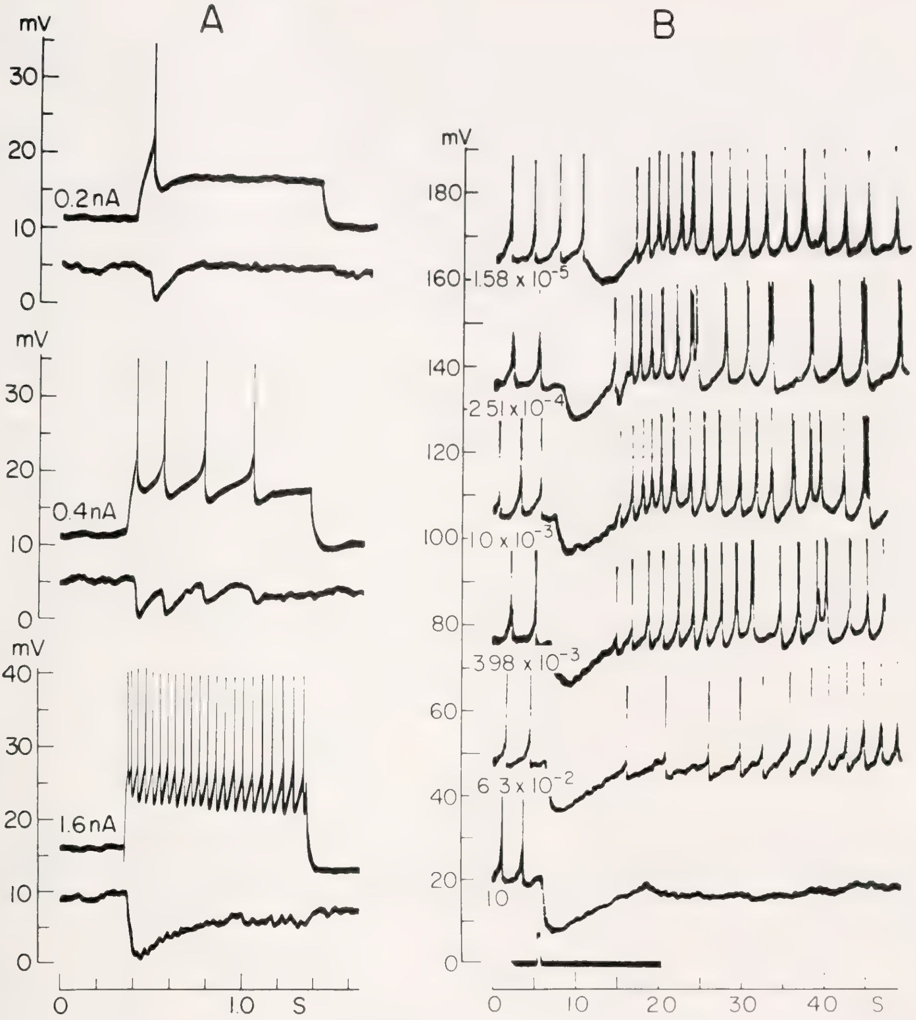


FIGURE 17A. Depolarizing currents to Type B photoreceptor produce impulses associated with IPSPs in simultaneously impaled optic ganglion cell (Alkon, 1973). B. Responses of "C" cell to light flashes of increasing intensity. Note increased frequency of firing after the hyperpolarization for dim to moderate flashes. The duration of hyperpolarization with cessation of firing increases only for the brighter flashes (Alkon, 1973). Values beneath intracellular voltage recordings refer to attenuation with neutral density filters of quartz-iodide source with intensity on preparation of 10^5 ergs$\cdot$cm$^{-2}\cdot$sec^{-1} designated as "1".

At least one type of optic ganglion cell (the "C" cell), recognized by its characteristic double spike, is inhibited by both ipsilateral and contralateral Type B photoreceptors (demonstrated by triple recordings in 12 different animals). Optic ganglion cells within the same optic ganglion do not have synaptic interactions (demonstrated with 25 pairs). Optic ganglion cells do interact, however, with other optic ganglion cells in the contralateral optic ganglion.

Neurochemical features of these photoreceptors and optic ganglion cells were also helpful in reproducibly identifying cells within the *Hermissenda* visual system. A number of observations, for instance, indicated a cholinergic basis for inhibitory

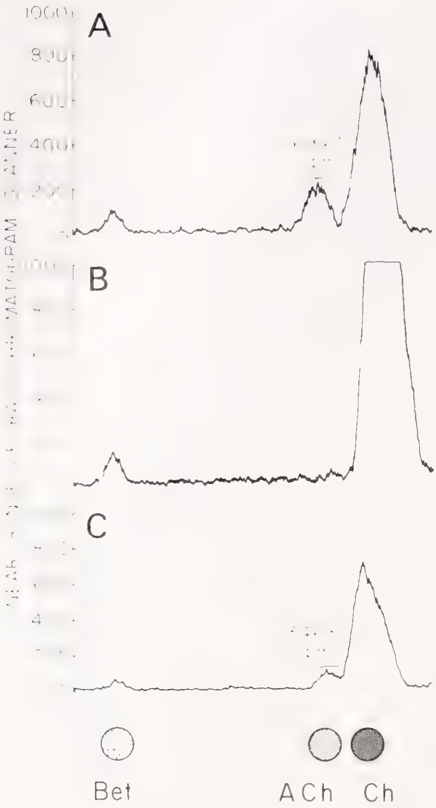


FIGURE 18. Radiochemical scan of electrophoretogram. A: combined sensory structures (eye, optic ganglion, statocyst) incubated with labeled choline. B: connective tissue incubated with labeled choline. C: eyes incubated with labeled choline. Ch, choline; ACh, acetylcholine; and Bet, betaine; labeled phospholipids (not shown) migrated very slightly (Heldman *et al.*, 1979).

interactions between the photoreceptors (Heldman *et al.*, 1979). The eyes, after isolation from the rest of the nervous system, synthesized and accumulated acetylcholine (as determined by electrophoretic separation of products that incorporated radioactive label) but not other putative neurotransmitter substances (Fig. 18). Electron micrographic (Crow *et al.*, 1979) sections showed clear round vesicles (consistent with synthesis and storage of acetylcholine) within the photoreceptors' somata, axon hillocks, and terminal branches. Pharmacologic experiments indicated cholinergic receptors on the terminal branches of the photoreceptors (Fig. 19). Neuronal endings within the optic tract, near the photoreceptor's terminal branches, stained for acetylcholinesterase. Another example of genetic specification of neurochemical characteristics was demonstrated within the optic ganglion. Using a histofluorescence technique to detect and localize small quantities of catecholamines or serotonin, we found that only one cell body within the optic ganglion fluoresced (Fig. 20). For 15/15 preparations this was clearly green long-lasting fluorescence (indicative of catecholamines) which appeared only after treatment of the freeze-dried ganglia with paraformaldehyde.

Hair cells

The statocysts of *Hermissenda* consist of 12–13 disk-shaped hair cells which are 40–50 μm in diameter and 5–10 μm in thickness. Scanning electron micrographs of normal specimens show the statocyst to be a sphere, 80–110 μm in diameter, surrounded by strands of a connective tissue bundle which also envelops each of the two *Hermissenda* eyes and optic ganglia. Each of the hair cells has approximately 120–150 modified cilia (motile hairs) (~ 8–10 μm long) which project into the lumen of the cyst and move a cluster of 150–200 crystalline structures known as statoconia (Figs. 21, 22).

Gravitational sense—that is, information about the direction of gravity relative to body position—is provided by the otolith organ in higher animals and by the statocysts of invertebrates. Both the statocyst and otolith organ consist of a cavity lined with mechano-sensitive hair cells; the cavity contains fluid and heavy (with respect to the density of the fluid) particles. A change in the direction of gravity causes the particles to move, in turn mechanically exciting the hair cells. Gravity effects on the *Hermissenda* statocyst can be simulated by rotating the isolated circumesophageal nervous system with intact statocysts (Fig. 22) while recording intracellular potentials from the statocyst hair cells. Hair cells located on the statocyst so as to be in front of the centrifugal force vector (Fig. 22) show an increased number and amplitude of small depolarizing waves (here termed "voltage noise"), depolarize, and increase firing in response to rotation. The hairs of these cells in

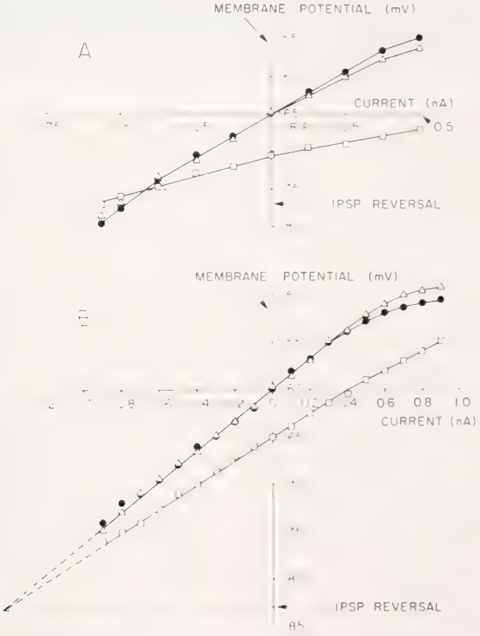


FIGURE 19. Photoreceptor current-voltage relations before and after perfusion with carbachol. I–V curves of Type A (A) and Type B (B) photoreceptors were plotted in control seawater (filled circles) and in presence of 10^{-4} M carbachol (open squares) for at least 10 min. The intersection of these two curves indicates the reversal potential of the drug-induced hyperpolarization. Recovery from the carbachol effect was seen 15 min after wash with seawater (open triangles). Arrows indicate the reversal potentials of the natural IPSPs in these same cells (Heldman *et al.,* 1979).

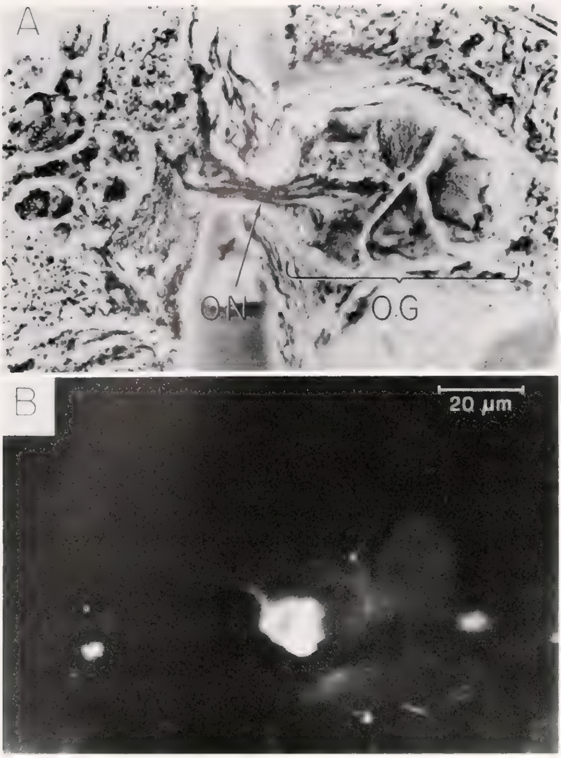


FIGURE 20. Formaldehyde-induced fluorescence in the optic ganglion (OG). A: phase contrast optics showing the ganglion and individual axons within the optic nerve (ON). B: fluorescence microscopy of the above. One cell within the optic ganglion has intense greenish fluorescence indicative of catecholamines. Note that the fluorescent material is concentrated in the cytoplasm and in the axon, but not in the nucleus (Heldman *et al.*, 1979).

front of the force vector experience the weight of the statoconia (as accelerated by rotation) and move with a fundamental frequency of 7 Hz. Hair cells located on the statocyst so as to be behind the force vector hyperpolarize, show a decreased voltage noise, and decrease firing in response to rotation. The hairs of these cells behind the force vector do not interact with the statoconia, which have been moved (as accelerated by rotation) to the opposite half of the statocyst (Fig. 22). These hairs move with a fundamental frequency of 10 Hz.

These observations and a number of additional experiments involving alteration of hair movement (with such treatments as cooling, hypertonicity, chloral hydrate, vanadate, and 4,4′-dithiobisphenyl azide) indicate that the hair cell generator potentials arise from summation of increased voltage noise (DeFelice and Alkon, 1977; Grossman *et al.*, 1978; Stommel *et al.*, in press) which in turn arises from force exerted by the statoconia on the hairs but not from hair bending *per se*. This force then causes sustained membrane distortion and consequent conductance changes at the base of the cilia (Fig. 23) rather than on the ciliary shafts.

The hair cells in *Hermissenda,* unlike vertebrate hair cells, have axons which join together to form the static nerve, which extends for 30–40 µm before entering the pleural ganglion. Cutting the nerve proximal to this point of entry can eliminate all spontaneous impulses and synaptic potentials (as was the case for cutting the photoreceptor axons) or those elicited by depolarizing current pulses. Thus, in an intact hair cell a depolarizing generator potential spreads passively from the hair cell's luminal surface and soma down its axon to an excitable zone where impulses are triggered. The impulses spread (probably in a regenerative fashion) into the hair cell's terminal branches, where they elicit synaptic potentials on neighboring hair cells and other post-synaptic neurons.

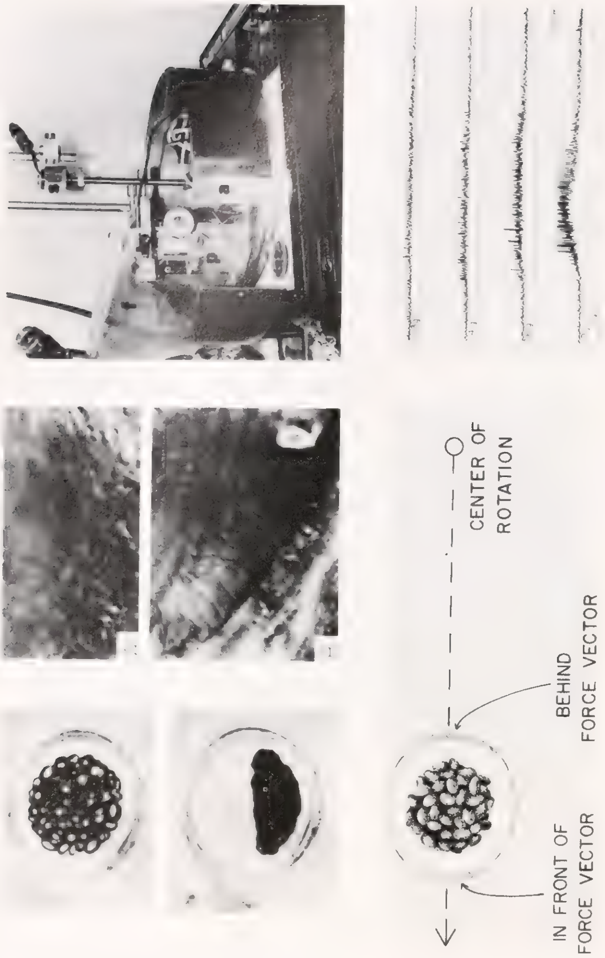
The synaptic organization of the statocyst enhances the differences in responses from hair cells located 180° with respect to each other on a statocyst equatorial locus. Thus, a kind of lateral inhibition provides increased contrast between gravitational responses of the hair cells for different orientations of the animal. Of 75 pairs of hair cells recorded within the same statocyst (Detwiler and Alkon, 1974) 43 pairs showed unidirectional inhibition, *i.e.*, stimulation of one cell of a pair caused inhibition of the other cell, but not vice versa. Unlike the IPSPs between photoreceptors or from Type B photoreceptors onto optic ganglion cells, this inhibition of a hair cell occurred only in response to a train of spikes in the presynaptic hair cell. Of these 43 pairs of hair cells, 36 were located at ~ 90° with respect to each other on statocyst equatorial loci. Of the 75 intracyst hair cell pairs, 16 showed reciprocal or mutual inhibition. The hair cells of all 16 pairs were located at ~ 180° with respect to each other on statocyst equatorial loci. Of the 75 intracyst pairs, 7 showed very weak electrical interaction. In 61 pairs of intercyst hair cells (*i.e.*, from each of the two statocysts), 15 showed substantial electrical coupling, 20 showed unidirectional or reciprocal inhibition, and 22 did not show any interaction.

Intersensory interactions

The same approach to investigating neural organization as described for photoreceptor, optic ganglion, and hair cell interactions, was also taken for interactions between the statocyst and visual pathways. In an initial study (Alkon, 1973a), I found that some hair cells inhibit ipsilateral and contralateral photoreceptors (11/59 photoreceptor-hair cell pairs). Type B photoreceptors inhibit ipsilateral and contralateral hair cells (4/36 pairs). Type A photoreceptors excite ipsilateral hair cells (3/17 pairs). Hair cells inhibit and are inhibited by ipsilateral optic ganglion cells (2/14 and 3/14 pairs). In subsequent studies (Tabata and Alkon, 1979, and in preparation) the nature of these intersensory interactions was correlated with the position of the hair cell on the statocyst. We found that only caudal hair cells (*i.e.*, on a dorso-ventral statocyst equator) inhibit the ipsilateral photoreceptors and Type B photoreceptor impulses can eliminate (Fig. 24) a source of tonic inhibition onto caudal hair cells from the ipsilateral optic ganglion. The presynaptic source of these IPSPs within the ipsilateral optic ganglion is also the source of EPSPs onto the ipsilateral Type B photoreceptor (Fig. 25). This presynaptic source of EPSPs and IPSPs is also inhibited by caudal hair cell impulses (Fig. 26).

Chemoreceptors

Agersborg (1922, 1925) originally presented histologic evidence for the location of chemoreceptors within the *Hermissenda* tentacles. Consistent with this evidence were our own preliminary histologic investigations (Fig. 8) and the finding that stimulation of the tentacle with food substances (squid extract, egg yolk) caused synaptic excitation of small neurons on the ventral side of the cerebropleural ganglion (CPG) (Alkon *et al.*, 1978). These EPSPs summated with


CENTER OF ROTATION
BEHIND FORCE VECTOR
IN FRONT OF FORCE VECTOR

higher concentrations of stimulus solutions to cause steady membrane depolarization and increased impulse activity of these neurons (11/26 cells). Glycine ($\geq 10^{-8}$ M) and DL-methionine ($\geq 10^{-10}$ M) solutions also caused EPSPs and depolarization of ventral CPG neurons. These ventral neurons were affected similarly by electrical stimulation of the nerve existing from the ipsilateral tentacle (44/55 cells). Other experiments showed that the photoreceptors, optic ganglion cells, and hair cells received synaptic inhibition from the tentacular chemosensory pathway.

Multisensory interneurons and motorneurons

In the pleural ganglion two classes of central neurons (Akaike and Alkon, 1980) were identified, which received input from the three sensory pathways just discussed: the visual, statocyst, and chemosensory. Of 74 neurons on the dorsal surface of the pleural ganglion (in a restricted region surround the optic tract's point of entry) 26 cells depolarized, 16 hyperpolarized, 4 depolarized and then hyperpolarized, and 28 cells showed no potential change in response to light. Discrete EPSPs were recorded from 14/26 neurons which depolarized and discrete IPSPs from 10/16 neurons which hyperpolarized in response to light. All of the discrete synaptic potentials followed impulses of ipsilateral Type B photoreceptors in a one-for-one fashion. Statocyst and chemosensory pathway stimulation produced on these central visual neurons synaptic effects which paralleled the effect of light. Thus, central neurons which received EPSPs (IPSPs) during light also, for most cases, received EPSPs (IPSPs) during statocyst and chemosensory pathway stimulation.

Our knowledge of the interneurons in these sensory pathways is still, of course, incomplete (Fig. 27). This is also true of putative motorneurons. Recently we have explored the largest cell (and two adjacent neighbors) in the *Hermissenda* nervous system. This cell, designated Pedal 1 for its location in the left pedal ganglion, receives synaptic input from the visual pathway and, upon electrical stimulation, controls movement of the cerata along the entire length of the animals (Jerussi and Alkon, 1980). In addition to mapping central ganglia motor neurons with intracellular electrodes we are also making extracellular records of impulse activity

FIGURE 21 (Sideways on page). Scanning electron micrograph of *Hermissenda* statocyst. (Upper) On the luminal surface of the statocyst, hairs make contact with statoconia. (Lower) Higher magnification of the above (Grossman *et al.*, 1979).

FIGURE 22 (Sideways). Upper left: Light micrograph of vital preparation of *Hermissenda* statocyst. Dorsal view (upper cyst) of equatorial cross-section shows statoconia maintained in a spherical cluster by hair movements. Same preparation after tilting microsocope 90° shows (lower cyst) statoconia moved by gravity to the lower half of the statocyst. Statocysts are ~ 100 μm in diameter. Upper center: (A) Vital preparation. Motile hairs in *Hermissenda* statocyst visualized with Nomarski optics (1,250 ×) in an intact statocyst placed on a microscope tilted 90°. Apical half of the motile hairs could not be photographed but could be visualized. (B) Preparation fixed with 0.5% glutaraldehyde. The full lengths of the hairs, now immobilized, are apparent in the photograph. A single statoconium (S) is seen among the hairs (Grossman *et al.*, 1979). Upper right: Photograph of modified Garrard turntable and recording apparatus (f) Faraday cage; (p) pedestal for preparation; (a) amplified; (s) slip rings. Lower left: Diagram illustrating the loci of hair cells with respect to a centrifugal force vector. Lower right: Response to rotation of hair cell with luminal surface opposite to the centrifugal force vector. The axon of the hair cell was cut to eliminate all synaptic but not impulse activity. Maximal centrifugal force is indicated at the left of each lower trace monitoring the rotation. The amplitude of monitor signal is proportional to the angular velocity of the turntable. The interval between monitor signals equals the period of the turntable's rotation. Impulse peaks are not included in the bottom record (Grossman *et al.*, 1979).

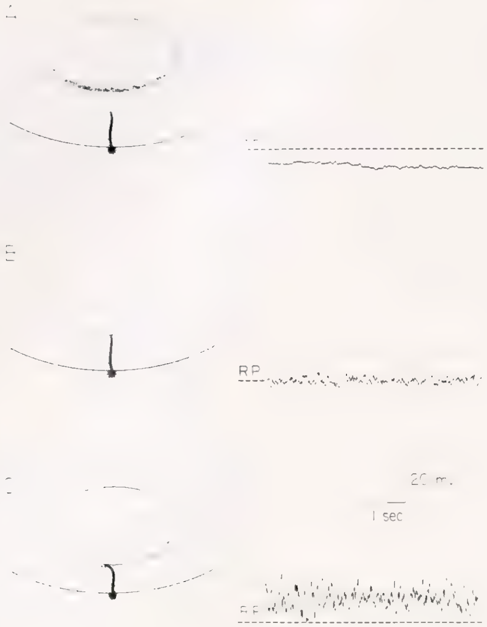


Figure 23. Conceptual model for hair-cell voltage-noise and generator-potential production. The hair is inherently motile (range of motion indicated by dash lines). (A) When the hair cell (oriented in the same direction as the centrifugal force vector) experiences a negative centrifugal force, the hair moves freely through a maximum range of motion producing little voltage noise (see noise record on right). (B) When the hair cell on that statocyst equator visualized when looking dorsoventrally is exposed to zero centrifugal force, the hair has some interaction with the statoconium. The statoconium exerts little net force on the hair although collisions of the statoconium with the rigid hair increase the voltage noise variance and frequency (voltage noise record on right). (C) When the hair cell (with luminal surface opposite the force vector) is exposed to a substantial centrifugal force (*e.g.* 1.0 g), the statoconium exerts a considerable net force on the hair, limiting its range of movement and reducing its movement frequency as well as dramatically increasing the voltage noise variance (see noise record on right). The increase of voltage noise variance and frequency results, by summation of individual events, in a depolarizing generator potential. Dash line indicates a positive centrifugal force exerted by rotation. All noise records were taken from actual hair cells under the conditions described (Grossman *et al.*, 1979).

within connectives exiting the circumesophageal nervous system and coursing peripherally to innervate the animal's musculature. We have been able to relate this output activity to input (light) responses of the photoreceptors (Richards, Lederhendler, and Alkon, in preparation). This approach should enable us to better understand the behavioral meaningfulness of changes in Type B firing frequency elicited by natural stimuli as well as consequent to training paradigms.

Neural Correlates of *Hermissenda* Learning Behavior

With an extensive, though by no means complete, knowledge of neural organization within and between *Hermissenda* sensory pathways, it was possible to ask what changes of this organization can be correlated specifically with the associative learning of the animal. More important, if such changes could be detected, it might also be possible to construct a causal sequence for the behavioral change beginning

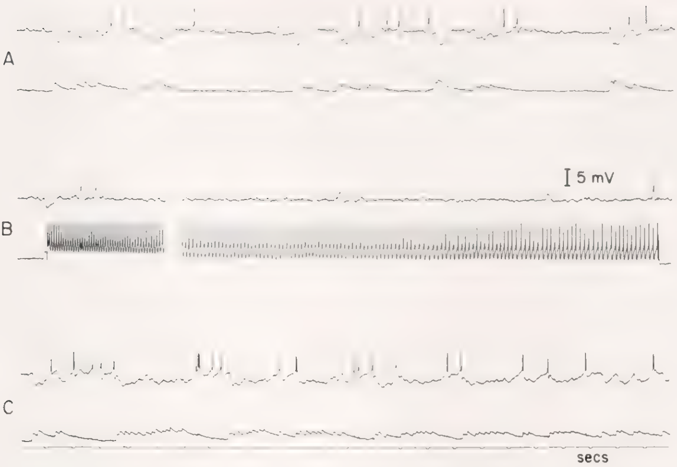


FIGURE 24. Simultaneous intracellular recordings of EPSPs from Type B photoreceptor (lower records in A, B, C) and IPSPs from ipsilateral caudal hair cell. Type B impulse (in response to 30.0 sec step at + 1.0 nA) eliminates IPSPs (B). EPSPs and IPSPs have increased frequently following current (C) (Tabata and Alkon, in prep.).

with the first neural site where the two distinct stimuli associated during training are associated by the animal's nervous system.

As a working hypothesis, a change at a well-defined convergence point between the statocyst and visual pathway seemed to be a reasonable primary neural locus where light paired with rotation could be associated by the *Hermissenda* neural systems. In fact, the first neural correlate I found of the short-term loss of *Hermissenda's* positive phototaxis following associative training was a marked reduction in excitation of ipsilateral hair cells by the type A photoreceptor (Fig. 28A, B). A subsequent study (Alkon, 1976) revealed that training with light paired with rotation (but not light alternating with rotation, *i.e.* unpaired presentation, nor light alone or rotation alone) caused the Type A cell to respond with far fewer impulses than during its depolarizing response to light alone (Fig. 29). This and other related observations suggested that the Type A cell was somewhat hyperpolarized following associative training because of increased inhibitory input from the Type B photoreceptors in the same eye (Alkon, 1976). Supporting this hypothesis was the finding (Alkon and Grossman, 1978) that even a single paired presentation of light and rotation was followed by a prolonged increase of Type B firing and an increased number of IPSPs onto the Type A cell as compared to unpaired or individual stimulus presentations (Fig. 30). These observations and their interpretation then suggested that the Type A photoreceptors would respond

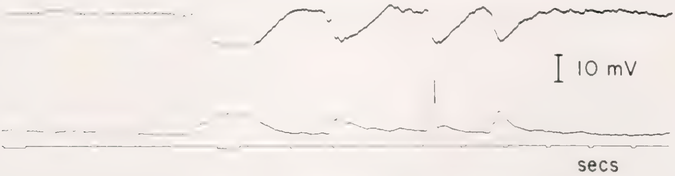


FIGURE 25. Simultaneous EPSPs (lower record) recorded from Type B photoreceptor and IPSPs (upper record) recorded from ipsilateral caudal hair cell. The presynaptic source of both types of synaptic potentials is within the ipsilateral optic ganglion (same cell as in Fig. 24).

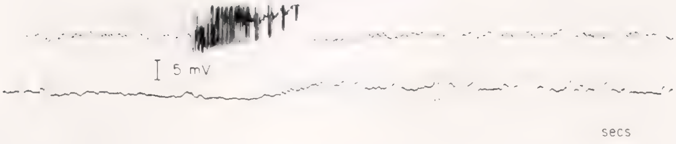


FIGURE 26. Depolarization of Type B photoreceptor (lower record) follows impulse train of ipsilateral caudal hair cell (upper record). A positive current pulse (+0.5 nA) injected into hair cell elicits increased firing of hair cell and slight inhibition of Type B cell. After high frequency firing hair cell remains slightly hyperpolarized and without impulse activity while Type B cells show increased EPSPs and prolonged depolarization (Tabata and Alkon, in preparation).

with fewer impulses and the Type B with more impulses in response to light during retention of long-term associative behavioral change specific to training with paired (but not random) light and rotation (Crow and Alkon, 1978). Intracellular recordings from nervous systems isolated from animals trained in this way showed that Type B photoreceptors' impulse activity following a light step was particularly enhanced in animals which had associative training ($\bar{X} = 2.84$), compared ($t = 2.47$; $P < 0.025$) to random ($\bar{X} = 2.09$) and other control training paradigms. These and other changes of Type B photoreceptor responses, specific to training with paired stimuli, also persisted during long-term retention of the associative behavioral change (Crow and Alkon, 1980). As another example of a change specific to associative training, Type B input resistance for the paired group ($\bar{X} = 88.5$ MΩ) was significantly greater (Mann-Whitney U test, $U = 4$; $P = 0.026$) than that for the random control group ($\bar{X} = 62.7$ MΩ). Dr. Crow and I also found that there was a significant positive correlation (Spearman $P = 0.63$; $P < 0.01$) between the Type B impulse frequency after training and the behavioral response latencies to

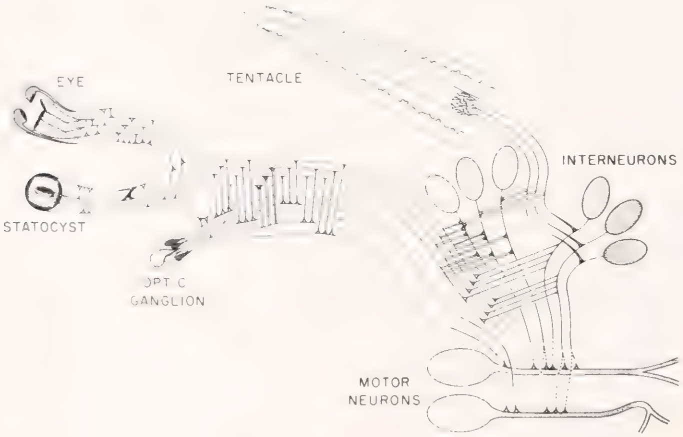


FIGURE 27. Schematic summary (partial) of ipsilateral neural interactions within and between the visual, statocyst, and chemosensory pathways. Branches perpendicular to lines (representing axons) indicate inhibitory synapses. Double-lined branches indicate excitatory synapses. Dash lines indicate synaptic interactions presently under study. No contralateral ipsilateral interactions (which have been investigated) are included.

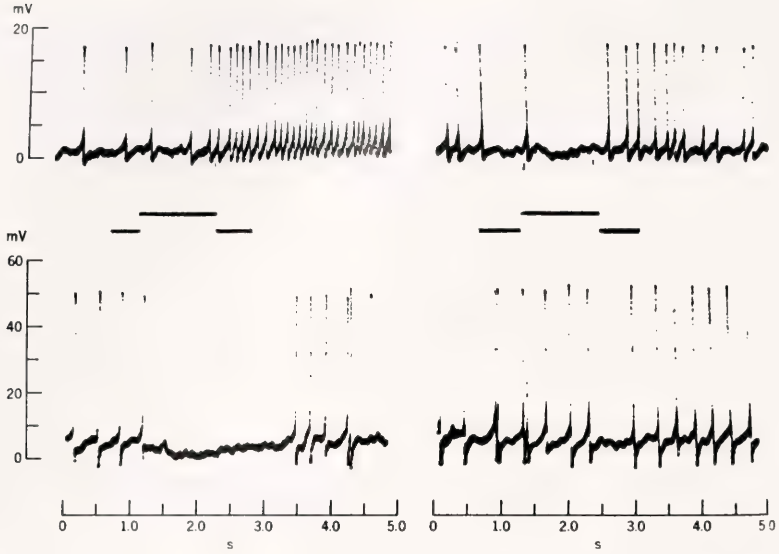
enter the light field for the paired light and rotation group (Crow and Alkon, 1978; Crow and Alkon, 1980). This last observation suggested a causal relationship between Type B impulse activity and the animal's associative behavioral change. Dr. Toshiaki Takeda and I have, in addition, observed similar changes following associative training in Type B photoreceptors' firing frequencies after a light step, which were correlated with and very possibly causal of changes in the firing frequency of the Pedal 1 cell (or putative motor neuron) after a light step (Takeda and Alkon, in preparation).

Cellular causality of associative learning

Long-term enhancement of Type B photoreceptor responses can account for the other observed neural changes (decreased hair cell excitation by Type A cells, decreased firing of Type A cells in response to light, decreased firing of Pedal 1 after a light step) produced by associative training. Since Type B firing is also known to control output activity in nerves exiting from the circumesophageal nervous system, these cells are very likely the site of a primary (if not the primary) neural change responsible for the associative learning of *Hermissenda.* If increased firing frequency of Type B cells has a primary causal role in the acquisition of the associative behavioral change retained by *Hermissenda,* it should not be due to a long-lasting increase in excitatory synaptic potential frequency. In fact, hyperpolarizing photoreceptors to the reversal potential of the IPSPs (between photoreceptors) did not reveal any difference in EPSP frequency between cells from the trained and random control animals (Crow and Alkon, 1980). Type B photoreceptors from the paired group also required a greater level of hyperpolarization from their resting level ($\bar{X} = 13.9$ mV) to block spike activity as compared with random controls ($\bar{X} = 7.6$ mV; $t = 4.66$; $P < 0.005$). These results together with other observations on Type B cells without impulses or synapses (see below) indicated that associative training at first results in a persistent non-synaptic depolarization of the Type B photoreceptors.

Such non-synaptic depolarization should also be observable when the same sensory stimuli, light steps paired with rotation, are presented repetitively to the isolated *Hermissenda* nervous system. Intracellular recordings during and after light and rotation stimulus regimens demonstrated that depolarization of type B photoreceptors following stimulus pairing (Fig. 31) accumulated with repetition. This accumulation was specific to pairing of light and rotation (*vs.* light alone, or explicitly unpaired stimuli) and to the orientation of the nervous system with respect to the center of rotation (Farley and Alkon, 1980; Alkon, in press). This cumulative depolarization following stimulus pairing did not occur when the circumesophageal nervous system was oriented with its cephalic pole way from the center of rotation (cephalic orientation). Indeed, for the cephalic orientation, paired light and rotation (but not explicitly unpaired stimuli) produced a slight hyperpolarization.

This cumulative Type B depolarization, specific to stimulus pairing and orientation, most likely represents the beginning of the long-term neural change which was observed after acquisition and during retention of the associative behavioral modification described above. If cumulative depolarization of the Type B cell is causally related to acquisition of the learned behavior, we would predict, on the basis of the intracellular recordings during training of the isolated nervous system (*cf.* Fig. 31), that restricting the animal to the caudal orientation should not prevent



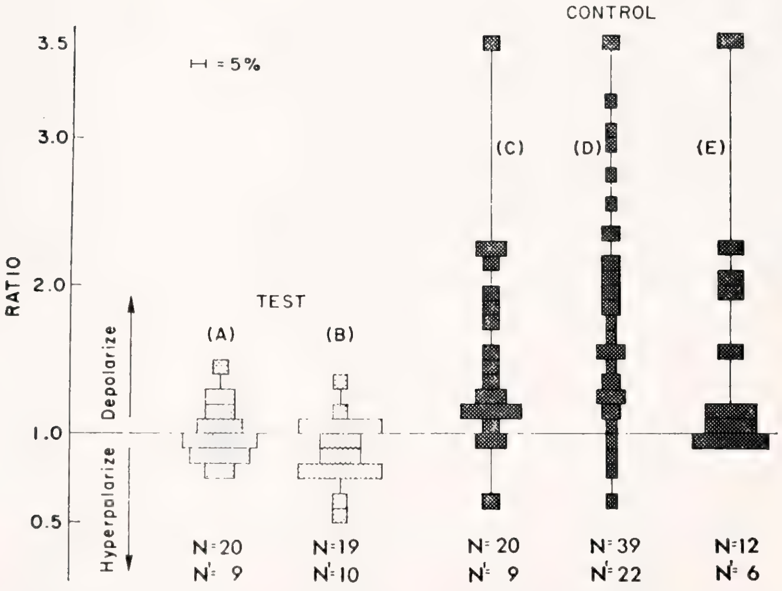


FIGURE 28A. Hair cell responses to light. Upper records are of hair cells in untrained animals. Left marker indicates duration of light flash to ipsilateral eye. Right marker indicates flash to contralateral eye. (Lower records) The hair cell of associatively trained animal responds with an isolated hyperpolarization to illumination of the ipsilateral eye, and shows no response with illumination of the contralateral eye. Intensity of flashes: 6×10^3 ergs·cm^{-2}·sec^{-1}. Base-line activity of lower hair cell: 210 impulses per min; of upper hair cell: 140 impulses per min (Alkon, 1975).

FIGURE 28B. Hair cell responses to illumination (6×10^3 ergs·cm^{-2}·sec^{-1}) of ipsilateral eye measured by the ratio of hair cell firing frequency for 10 sec immediately after to the frequency for 10 sec immediately before a 1-sec test flash. Each measurement was the average of two to four responses. Test group A, B: Animals exposed to 3 hr of rotation associated with light; A: maintained with 6.3 hr of daily light. B: maintained with 18 hr of daily light. Control group C: Animals exposed to 3 hr of rotation in darkness, maintained as Group A. Control group D: Animals taken directly from aquarium, maintained as Group A. Control

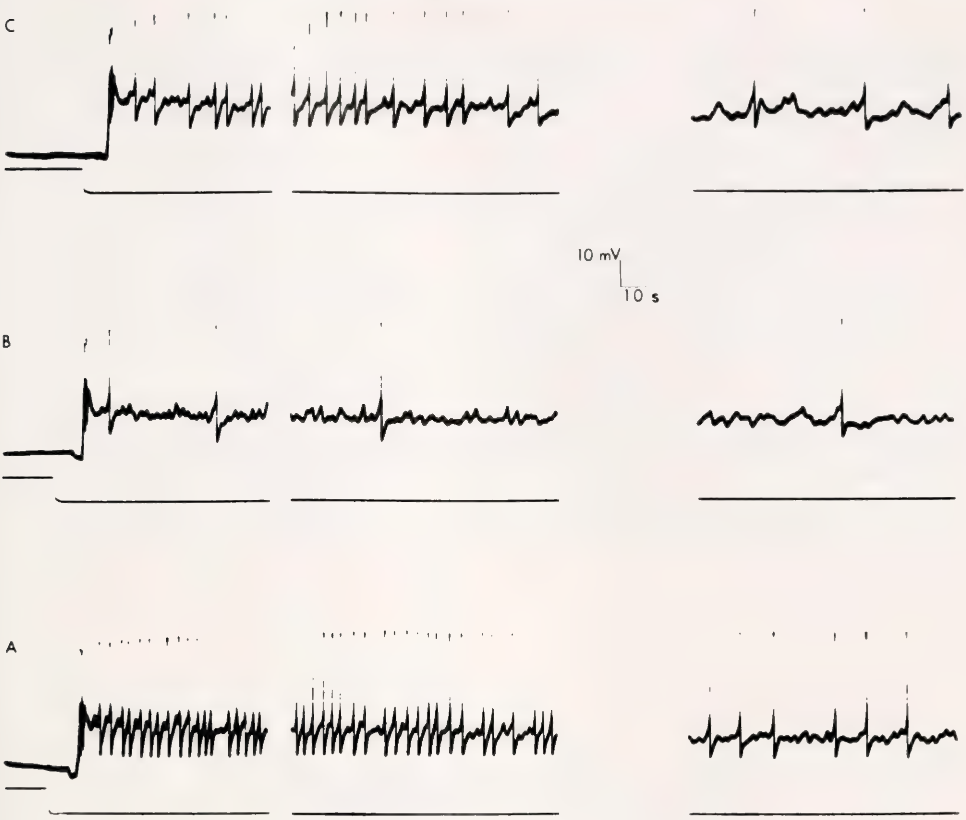


FIGURE 29. Effect of rotation and light stimulus regimen on responses of type A photoreceptor to light steps. A. Steady-state response before stimulus regimen. B. Response immediately after stimulus regimen. C. Response 20 min after stimulus regimen. Bottom trace in each record indicates duration of the light stimulus. Note that the primary effect of stimulus regimen is a decrease of firing frequency during the type A response. This effect is reduced 20 min after stimulus regimen. Spike amplitudes in B are slightly smaller in comparison to spike amplitudes in A and C for approximately equal firing frequencies. First two frames in A, B, C interrupted by 1 sec. Second and third frames interrupted by 20 sec (Alkon, 1976).

the training effects and might even exaggerate them. We would also predict that restricting the animal to the cephalic orientation will prevent training effects and may even reverse them. These predictions were confirmed by behavioral experiments (Farley and Alkon, 1980, and in preparation).

Although incomplete, the evidence for the Type B photoreceptor's primary responsibility for *Hermissenda's* associative learning is quite substantial. The evidence for a change located within this cell's soma was complete and unequivocal. Dr. Terry Crow and I measured increased input resistance (Fig. 32), membrane depolarization, and facilitated light responses which were specific to associative training (Crow and Alkon, 1978; Crow and Alkon, 1980) even after the cell's axon

---

group E: Animals, maintained as Group A, exposed to 3 hr of light intervals (identical to those used for the test groups but without associated rotation). Horizontal extent of bars is proportional to the percentage of animals within a given ratio interval. N = Number of hair cells. N′ = Number of animals (Alkon, 1975).

TABLE I

Comparison (Mann-Whitney U test) of membrane characteristics for Type B cells (cut nerve)

Characteristic	Paired *vs.* Random	
	(*U*)	(*P*)
Input resistance	10	0.025
Membrane potential	5	0.024
Hyperpolarizing phase of light response	5	0.024
Long-lasting depolarization	7	0.053

and terminal branches had been removed by a razor cut (Table I). After such a cut the Type B cell is electrically isolated from all other cells in the *Hermissenda* nervous system (Fig. 35; Alkon, 1979). This is so because the photoreceptors are not electrically coupled and because no chemical synapses occur on the somata. Thus, these changes could not be explained as secondary to changes of synaptic

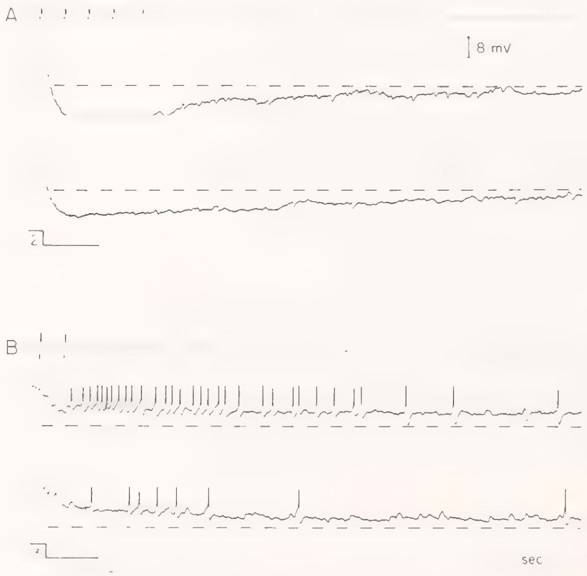


FIGURE 30. Effect of paired light and rotation stimuli on Type A and B photoreceptor responses. A. Responses of intact lateral type A photoreceptor to light alone (lower record) and light paired with rotation (upper record). The end of a 10-sec light step (intensity in − log units) is indicated by bottom trace. The end of a rotation stimulus (onset 5 sec before light), with a maximum of 1.3 *g*, is indicated by top trace. The number of IPSPs during the LLH (long-lasting hyperpolarization) is greatly increased by paired stimulation. Maximum hyperpolarization is also greater after light step but before rotation has ceased entirely. B. Responses of intact type B photoreceptor to light alone (lower record) and light paired with rotation (upper record). The end of a 20-sec light step (intensity in − log units) is indicated by bottom trace. Rotation, indicated by top trace, as above. The number of impulses during the LLD is greatly increased by paired stimulation (Alkon and Grossman, 1978). These recordings were made with a brush pen recorder (frequency response to 40 Hz). Note the Type A recording in A saturates the pen's maximum range following stimulus pairing.

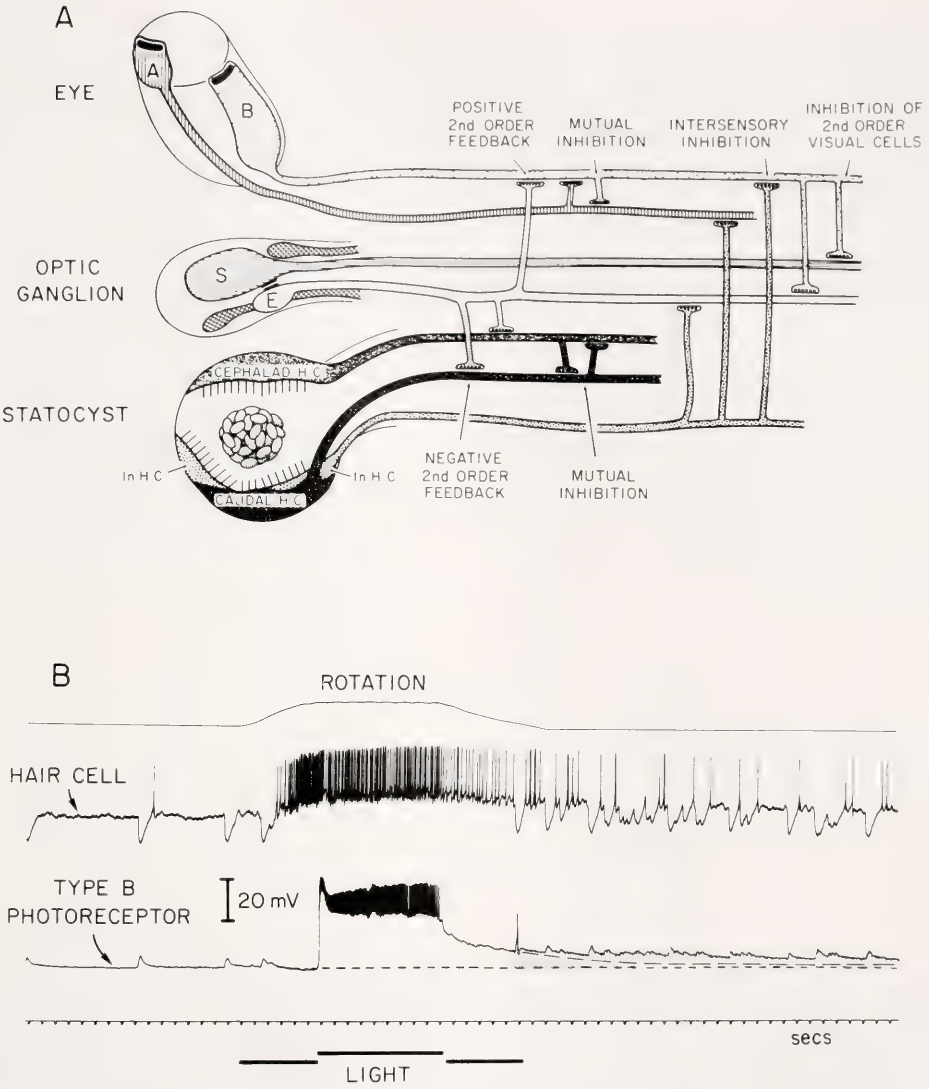

A
EYE
A
B
POSITIVE 2nd ORDER FEEDBACK
MUTUAL INHIBITION
INTERSENSORY INHIBITION
INHIBITION OF 2nd ORDER VISUAL CELLS
OPTIC GANGLION
S
E
STATOCYST
CEPHALAD H C
In H C
In H C
CAUDAL H C
NEGATIVE 2nd ORDER FEEDBACK
MUTUAL INHIBITION
B
ROTATION
HAIR CELL
TYPE B PHOTORECEPTOR
20 mV
secs
LIGHT

FIGURE 31. Neural responses to stimulus pairing. (A) Neural system (schematic and partial diagram) responsive to light and rotation. Each eye has two type A and three type B photoreceptors; each optic ganglion has 13 second-order visual neurons; each statocyst has 12 hair cells. The neural interactions (intersection of vertical and horizontal processes) identified to be reproducible from preparation to preparation are based on intracellular recordings from hundreds of pre- and post-synaptic neuron pairs. Abbreviations: In HC, hair cell ~45° lateral to caudal north-south equatorial pole of statocyst; S, silent optic ganglion cell, electrically coupled to E cell; E, optic ganglion cell, presynaptic source of EPSPs in type B photoreceptors. The E second-order visual neuron causes EPSPs in type B photoreceptors and cephalad hair cells and simultaneous inhibitory postsynaptic potentials (IPSPs) in caudal hair cells. (B) Intracellular recordings (simultaneous) from caudal hair cell and type B photoreceptor shows increase of EPSP's (type B cell, lower record) and simultaneous IPSPs (caudal hair cell, upper record) after light paired with rotation. The LLD after stimulus pairing is greater than that after light alone (line of long dashes). The line of short dashes indicates level of resting membrane potential. The lowest trace indicates light duration; top trace, angular velocity of turntable (effecting 1.2 *g*) (Alkon, 1979).

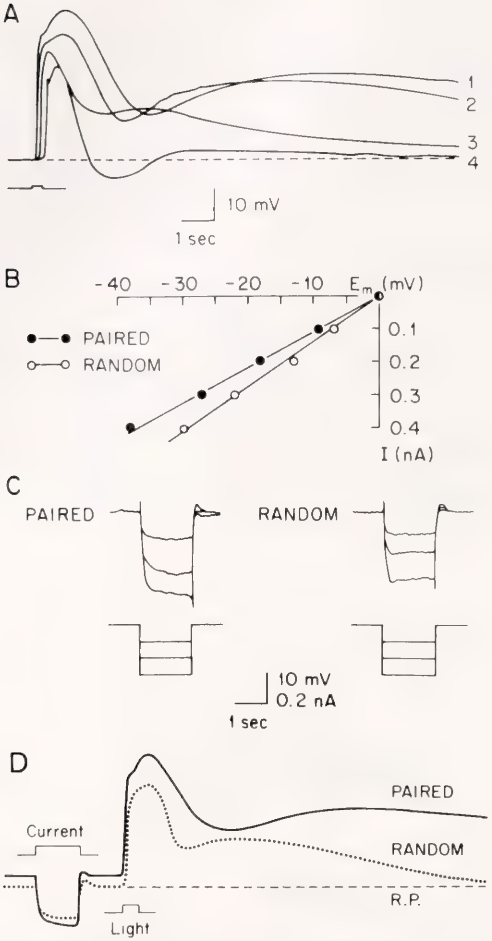
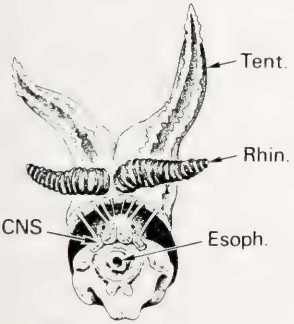


FIGURE 32. Cellular changes in cut nerve preparations from experimental and random control animals. (A) Receptor potentials of dark-adapted Type B photoreceptor from an experimental animal (light paired with rotation). Responses evoked by brief light flashes of increasing intensity (4), − log 3.0; (3), − log 2.0; (2), − log 1.0; − log 0.5. Dash line indicates resting membrane potential and lower trace indicates duration of light flash. The absence of spikes and synaptic potentials indicate that the photoreceptor soma was successfully isolated from the area of spike initiation and synaptic input. (B) Representative linear current-voltage relationship of dark-adapted isolated (cut nerve) Type B photoreceptors from experimental (paired) and random control groups. (C) Examples of changes in membrane potential of dark-adapted isolated B photoreceptors from experimental and random control animals. Electrotonic potentials evoked by hyperpolarizing square current pulses (bottom traces) through a balanced bridge. Resistance measurements taken with the single electrode-bridge circuit are consistent with data from experiments in which the photoreceptors were impaled simultaneously with two microelectrodes for current injection and voltage recording (Crow and Alkon, 1980). (D) Voltage representation of membrane changes during associative learning. Theoretical voltage recordings of Type B (cut nerve) responses to light following three days of paired and randomly associated light and rotation. These responses are based on data referred to in Table I and actual responses of Fig. 32A, B, C.

input, for instance, from the optic ganglion (see above). Furthermore, since the Type B cell is at the input stage of the *Hermissenda* nervous system, it can be expected to affect post-synaptic neurons at various stages within the visual pathway.

A quantitative assessment of the degree to which a change in Type B cell responses to light causes a change in output impulse activity and motor responses certainly will require further extensive investigation. Additional evidence will be provided by recordings from cells of intact (Jerussi and Alkon, 1980) and semi-intact (Fig. 33) behaving animals. Elimination of Type A cell activity by negative current injection might reduce or eliminate the behavioral response to light (as occurs with increased impulse activity of Type B cells and the consequent increased inhibition of the Type A cells). Hyperpolarization of the Type B cell after associative training should help restore the normal behavioral response to light. Similar experiments at each station of the visual pathway of behaving animals will further define the causal role of known neural elements such as the Type B photoreceptors in the visual behavior and its modification by training.

The difficulties of precisely defining processes which cause changes of animal behavior is illustrated by work on habituation of the gill withdrawal reflex of *Aplysia*. Although it has been possible to recognize some large identified neurons in the *Aplysia* abdominal ganglion, a comprehensive description of neural circuits which entirely account for even this relatively simple reflex and its habituation has never been completed (Jacklet and Rine, 1977). Thus, although the excitatory postsynaptic potentials (EPSPs) produced by sensory cells on motor neurons are known to be responsible for much of this behavior (Castellucci *et al.*, 1970), peripheral



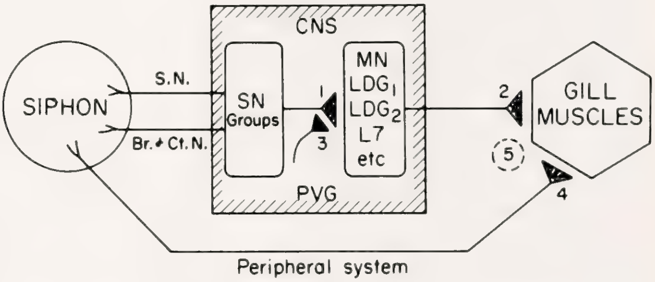


FIGURE 33. (Left) Semi-intact preparation of *Hermissenda* circumesophagael nervous system. The visual, statocyst, and chemosensory pathways are intact although most of the animal's foot has been removed.

FIGURE 34. (Right) Diagram of the synaptic sites for habituation of the gill withdrawal reflex activated by tactile stimulation of the siphon. Sensory information is conveyed to the central nervous system (CNS) via the siphon nerve (S.N.) and other nerves (Ct.N and Br.N) to central sensory neurons (SN). The synapses (site 1) of sensory neurons with motor neurons (MN) are subject to synaptic depression and the synapses (site 2) of motor neurons with gill muscles are subject to frequency-dependent homosynaptic facilitation. Dishabituation causes heterosynaptic facilitation of the sensory neuron terminals (site 3) and the firing of motor neurons, which facilitates neuromuscular junctions (site 2). The facilitation and its subsequent decay is frequency-dependent and may carry over from trial to trial. Additionally, the gill is activated (most effectively in the absence of the PVG) by a peripheral pathway (15) that shows habituation and dishabituation, presumably at chemical synapses (site 4). Other synaptic influences of excitatory or inhibitory sign and central or peripheral origin (site 5) may also influence habituation. For example, inhibitory junction potentials in gill musculature (14) and the suppressive influence of the PVG on the peripheral pathway (15) may contribute. Also, the branchial (gill) ganglion, which is important in gill habituation to direct gill stimulation (21, 22) may contribute. Responses of the isolated siphon to direct siphon stimulation also readily habituate (23–25) (Jacklet and Rine, 1977).

neuronal interactions have also been implicated (Peretz, 1970; Peretz and Estes, 1974). Interneurons which have interactions with both sensory and motor neurons are thought to exist (Fig. 34), but their influence has not been fully assessed. Interactions between sensory cells and between motor neurons also have not been rigorously analyzed. The close correlation of EPSPs (recorded from the motor neurons) and behavioral decrement during habituation of the gill withdrawal reflex has been observed by Kandel and his colleagues (Carew *et al.,* 1972; Carew and Kandel, 1973). Other studies revealed, however, that central ganglionic neuronal changes were not alone sufficient to explain habituation and that peripheral neuronal changes had to be included (Peretz, 1970; Bruner and Kennedy, 1970; Lukowiak and Jacklet, 1972; Peretz and Howieson, 1973; Lukowiak and Peretz, 1974; Peretz *et al.,* 1976; Jacklet and Rine, 1977).

VOLTAGE-DEPENDENT CONDUCTANCES AND ASSOCIATIVE LEARNING

To better understand the ionic basis of the observed changes within the soma membrane of the Type B photoreceptor during associative learning of *Hermissenda* and to examine how stimulus pairing is specifically encoded by the effects of synaptic interactions on this cell's ionic conductances, voltage-clamp analysis in our laboratory was undertaken. The Type B photoreceptor, located in the anterodorsal portion of the eye, is 35 μm in diameter. Its axon, ~ 1 μm in diameter, leaves the base of the eye, enters the optic nerve, passes ensheathed through the optic ganglion (for ~ 50 μm), enters the optic tract, and terminates in a spray of fine endings ~ 20 μm from its entry into the cerebropleural ganglion (Fig. 13). The synaptic interactions and impulses described only occur along the distal axon and terminal endings. Thus, cutting through the nerve near the impulse-generating zone leaves a cell body that contains the phototransduction apparatus without impulses or synaptic interactions (Fig. 35). Since, following lesion, input resistance tends to increase slightly, significant shunting of currents injected or measured across the soma membrane is unlikely. Voltage- and current-clamp recordings can then be made with two electrodes in the cell body under favorable space-clamp conditions.

Type B photoreceptors in cut-nerve preparations depolarize for 1–2 min after a 30-sec light step of moderate intensity of 510 nm (10^3–10^4 ergs$\cdot$cm$^{-2}\cdot$sec^{-1}). This long-lasting depolarization (LLD) is always associated with a membrane resistance 1.5–3 times higher than the resting or dark value. Positive current injection, causing depolarization comparable to that produced by light, is not followed by an LLD or increased membrane resistance (Fig. 36). With sufficient hyperpolarization during the light response, however, the LLD and its associated conductance decrease could be eliminated. These observations suggested that the LLD arises at least in part from a voltage-dependent decrease of K^+ conductance within the Type B soma membrane. Furthermore, since the LLD increased when the light step was paired with rotation, this increase with pairing could be due to the LLD's voltage-dependence.

Voltage-clamp experiments provided additional insight into the nature of voltage-dependent conductances of the Type B soma membrane. In darkness a voltage-dependent increase of total membrane conductance (Fig. 37) was apparent with steady-state changes of holding potential from resting (~ -55 mV) to more positive levels (Alkon, 1979). Step clamp commands (Shoukimas and Alkon, 1980) from a holding potential of -60 mV elicit an early, transient outward current which appears to be carried predominantly by K^+ ions. Both steady-state ac-

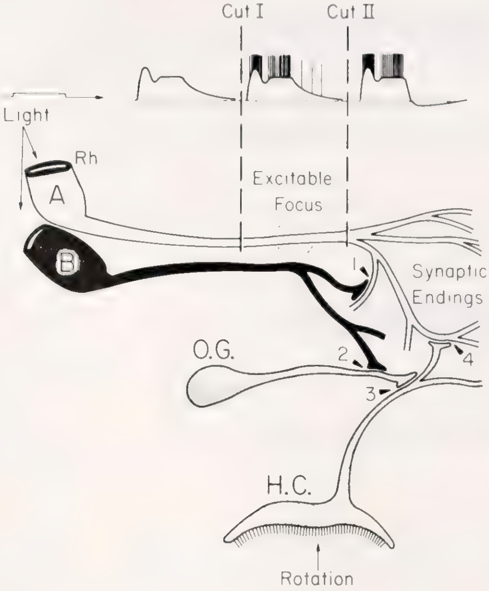


FIGURE 35. Excitation and inhibition of type A photoreceptor. Diagram of type A photoreceptor soma with rhabdome, axon, excitable focus, and terminal branches. Light depolarized photoreceptors at rhabdomes (Rh). Type B photoreceptors (of which there are three) inhibit, at synaptic endings, lateral type A, directly (1) and indirectly by inhibiting (2) optic ganglion (O.G.) cells (of which there are 13). Ipsilateral hair cells (H.C.) receive less inhibition from optic ganglion cells (3) when the type B fires and thus increases its inhibition of the lateral type A (4). Hair cells also inhibit type A when they depolarize in response to rotation. In response to light, the type A cell depolarizes without impulses or after-hyperpolarization with cut I lesion. It depolarizes with impulses but without after-hyperpolarization with cut II lesion. The response of an intact type A cell is represented at upper right (Alkon and Grossman, 1978).

tivation and inactivation of the conductance are voltage-dependent. Unlike some other gastropod neurons in which similar currents have been reported, such as *Anisodoris* (Connor and Stevens, 1971), the kinetics of inactivation, like the steady-state value, show voltage-dependence.

Voltage-dependent conductances were also indicated during and following light steps (as well as in darkness as described above). In brief, the three currents induced during a light step were most likely carried by:

1. Na^+, a rapidly decreasing inward current, greatly reduced by replacing external Na^+ with equimolar tetramethyl ammonium ions (TMA) and reversing at $\sim +60$ mV (Fig. 37A).

2. K^+, a rapidly decreasing outward current, greatly reduced by 4 mM external 4-aminopyridine, intracellular injection of tetraethyl ammonium ions (TEA) and ethylene-glycol tetraacetic acid (EGTA) and 1 mM external Ni^{2+}, and reversing at ~ -80 mV. The reversal potential became more positive when external K^+ was raised (Fig. 37, 38).

3. Ca^{2+}, a sustained inward current that decreased slowly following the cessation of the light step, was present in Na^+-free artificial sea water (ASW), reduced by lowering external Ca^{2+}, increasing external Mg^{2+}, and 1 mM external Ni^{2+} (Figs. 37; 38B, C; 39). This current was reduced by intracellular injection of

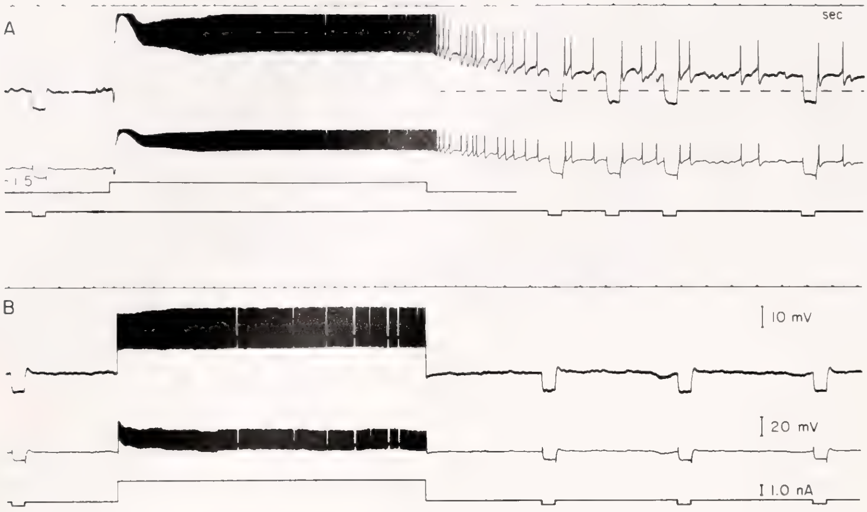


FIGURE 36. Effect of light and positive-current steps on intact type B photoreceptor. Cell was impaled simultaneously with two microelectrodes: one to measure voltage only, the other to measure voltage as well as to inject current (lower voltage traces in each pair). A: LLD associated with decreased membrane conductance following a 30-sec light step (indicated by lowest trace and intensity expressed in − log units). Membrane conductance was measured by injection of negative-current pulses (indicated by lowest tract). Dash line indicates level of resting membrane potential. B: Response to injection of positive-current step, 1.5 nA. Membrane conductance, measured by injection of negative current pulses (indicated by lowest trace), increases during positive current (delayed rectification). Note the brief hyperpolarizing wave following current step. Pulses are − 0.5 nA. Note changes of paper speed in A, B (Alkon and Grossman, 1978).

cyclic-AMP, but not by TEA, and was increased by injection of EGTA. This Ca^{2+} current was markedly voltage-dependent, not appearing at holding potentials $< \sim -35$ mV and reversing at holding potentials $> +100$ mV. With elevated external K^+ (120 mM) the Ca^{2+} current was still observed at the approximate reversal potential for the light-induced K^+ current.

After a light step there was a prolonged outward current. This current increased with holding potentials more positive than the resting potential (-55 mV) and decreased with more negative holding potential in ASW and Na^+-free ASW. This late outward current was eliminated by 4 mM 4-aminopyridine (Fig. 39), intracellular TEA and EGTA injections, and 1 mM Ni^{2+} added to ASW. These data indicate that the outward current at holding potentials $\geq$ R.P. is a K^+ current which depends on a light-induced Ca^{2+} current. Following a light step a substantial prolonged Ca^{2+} current (most apparent in 4-aminopyridine, which blocks the late outward current) will only occur at holding potentials > R.P. The light-induced K^+ current(s), therefore, can be enhanced by a light-induced increase of intracellular Ca^{2+} only at holding potentials > R.P.

How can these voltage-dependent conductances together with the known synaptic interactions encode stimulus pairing and ultimately result in a long-lasting nonsynaptic depolarization of the Type B photoreceptor? Because of the synaptic interactions within and between the visual and statocyst pathways (Fig. 31), rotation paired with light is followed by disinhibition of the Type B cell and an increased number of EPSPs in this cell. Because membrane resistence (and thus the membrane time constant) is increased following a light step, the synaptic depolarization (due to disinhibition and the EPSPs) is enhanced. The synaptic depolarization following stimulus pairing, however, also facilitates the light-induced depolarization that was shown to arise from voltage-dependent Ca^{2+} and K^+ conductances.

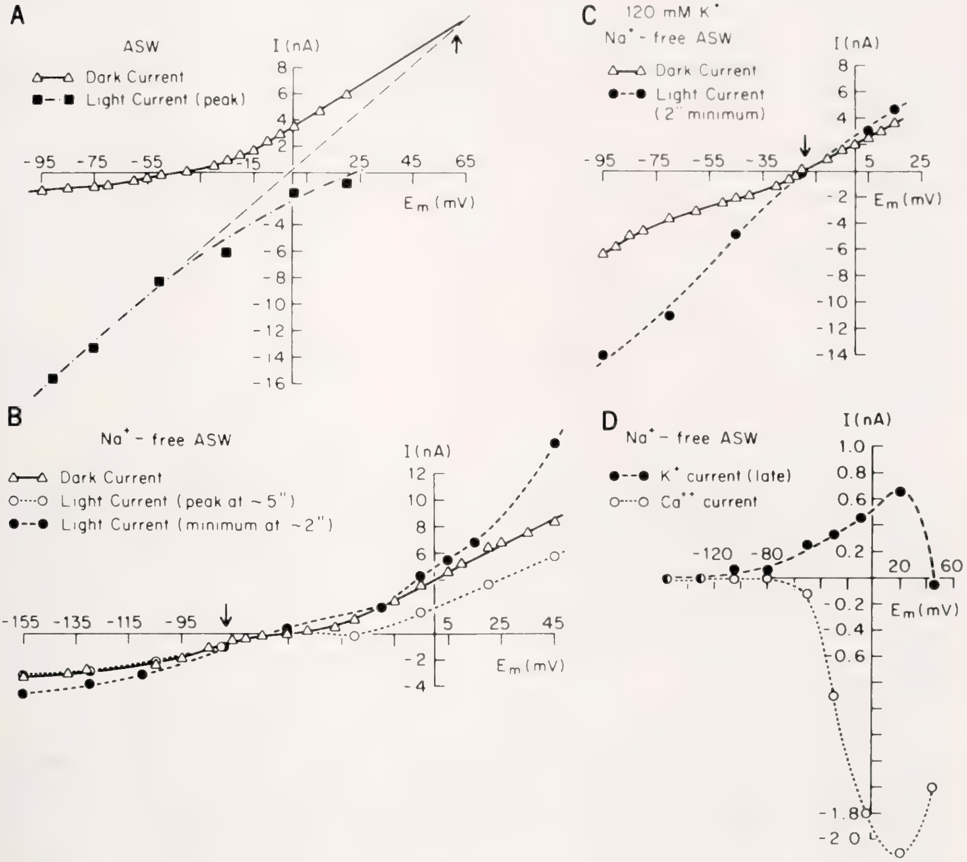


FIGURE 37. Current-voltage plots from voltage-clamp recordings in darkness and during and after light steps (unfiltered); negative current = inward current. (A) Dark current and maximum initial inward current (Na^+) in ASW in response to 1.8×10^6 erg·cm^{-2}·sec^{-1} at different holding potentials. Arrow indicates reversal potential extrapolated from linear portion of current-voltage of I–V plot of Na^+ current. (B) Dark current and isochronal (2 and 5 sec after light onset) values of initial outward current (K^+) and sustained inward current (Ca^{2+}) in Na^+-free ASW in response to 1.8×10^6 and 1.1×10^6 erg·cm^{-2}·sec^{-1}, respectively, at different holding potentials. There is no measurable sustained inward current at holding potentials < RP. (C) Dark current and isochronal (2 sec after light onset) value of initial outward current (K^+) in 120 mM K^+ in Na^+-free ASW in response to 1.8×10^6 erg·cm^{-2}·sec^{-1} at different holding potentials. Reversal potential (arrow) of outward current has shifted toward more positive holding potential. (D) Absolute values of sustained light-induced inward current (Ca^{2+}) and late outward current (K^+) as a function of holding potential. Ca^{2+} value was measured 3 sec after light step cessation; K^+ value, 35 sec after light step cessation. Note the parallel relation of the two currents to holding potential. Light intensity, 1.1×10^6 erg·cm^{-2}·sec^{-1} (Alkon, 1979).

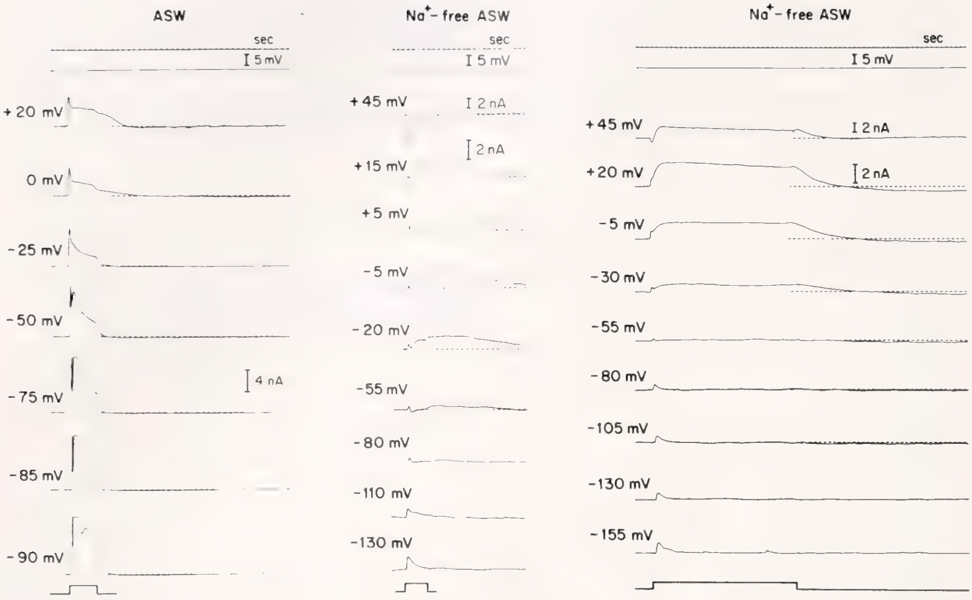


FIGURE 38. Voltage-clamp recordings during and after light steps; up = inward current. Values at left are absolute holding potentials. Dash lines indicate steady-state current in darkness for each holding potential. Voltage traces at top were recorded simultaneously with current (flowing from reference element in the bath) recordings. Lowest trace in each set indicates duration of light step. A sustained inward current (Ca^{2+}) is induced by light at holding potentials > RP in Na^+-free ASW. The early outward current (K^+) is marked at higher light intensity (middle column). Light intensity: in middle and left column: 1.8×10^6 $erg \cdot cm^{-2} \cdot sec^{-1}$ (unfiltered); in right column: 1.1×10^6 $erg \cdot cm^{-2} \cdot sec^{-1}$ (unfiltered). Paper recording amplifier saturates for some records in ASW (Alkon, 1979).

Thus, a kind of regenerative or positive feedback mechanism is suggested as a neurophysiologic basis for the associative learning of *Hermissenda*. Light-induced depolarization enhances synaptic depolarization, which in turn enhances the light-induced depolarization, and so forth. With each successive trial, residual depolarization could potentiate and add to depolarization following the next stimulus pair. How does cumulative depolarization (Farley and Alkon, 1980; Alkon, in press) of the Type B membrane, in turn, cause a very long-lasting change which could account for the animals' decreased positive phototactic behavior? A causal sequence which is consistent with the currently available data is as follows. Cumulative depolarization, specific to stimulus pairing, causes inactivation of dark voltage-dependent K^+ conductances (*cf.* Shoukimas and Alkon, 1980). Inactivation of voltage-dependent K^+ conductance(s) causes increased total membrane resistance of the Type B membrane when it is depolarized in response to a light step. An increased membrane resistance would be associated with greater depolarization (of the Type B cell) resulting from light-induced ionic currents. Greater depolarization of the Type B cell in response to light causes facilitation of the voltage-dependent light-induced Ca^{2+} currents.

In brief, cumulative depolarization (following associative training) causes inactivation of a dark K^+ conductance and facilitates the light-induced responses of the

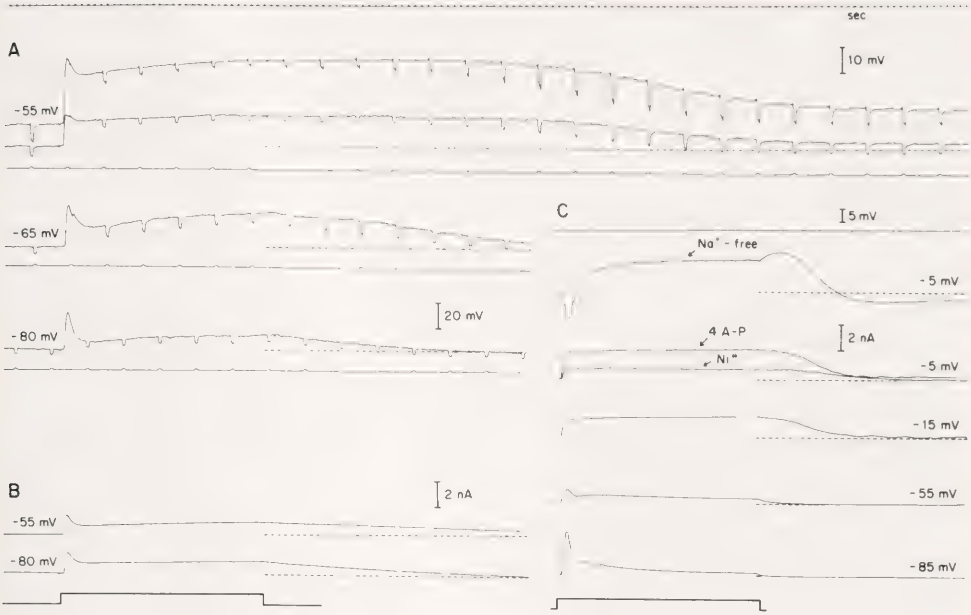


FIGURE 39. Effects of blocking agents on voltage and voltage-clamp recordings during and after light steps (A) Voltage recordings from type B cell in ASW containing 1 mM $NiCl_2$. Two microelectrodes recorded voltage simultaneously (upper two recordings). Current pulses injected through one microelectrode via a bridge circuit caused smaller voltage change during light (compared to before light) and transiently a larger voltage change after the light (compared to voltage change before light); this indicates increased membrane resistance after light (compared to membrane resistance before light). The LLD after light persisted in $NiCl_2$ when membrane resistance was not elevated. This was not so without $NiCl_2$. The LLD was reduced at more negative potentials (produced by injecting steady negative current through one microelectrode); light intensity (indicated by bottom trace), 1.1×10^6 erg·cm^{-2}·sec^{-1} (unfiltered). (B) Voltage-clamp recordings from same cell as in (A) under same conditions at comparable holding potentials; up = inward current. Note the long-lasting inward current after light step. Voltage traces at top were recorded simultaneously with current recordings. (C) Voltage-clamp recording during and after light steps in presence of blocking agents. Voltage traces at top were recorded simultaneously with current recordings. Upper recordings in Na^+-free ASW, 1 mM 4-aminopyridine (4 A-P), and 1 mM $NiCl_2$ were all at − 5 mV absolute holding potential. Current records at lower holding potentials were for same cell in 4-aminopyridine. Note absence of measurable early or late outward currents during and after light (intensity, 1.8×10^6 erg·cm^{-2}·sec^{-1}, unfiltered), and presence of slowly decreasing sustained inward current (Ca^{2+}). Dash line indicates steady-state current level in darkness (Alkon, 1979).

Type B cell. The Type B cell with its enhanced response to light now, via synaptic inhibition, more effectively reduces the Type A cell impulse activity in response to light and this finally is expressed as a decreased positive phototaxis.

Conductance changes in non-gastropod learning models

Woody and his colleagues observed increased excitability of cells in the coronal pericruciate cortex to current injection in cats conditioned to eye blink by a Pavlovian training procedure (Woody *et al.*, 1974). Woody and Black-Cleworth (1974) proposed that increased neuronal input resistance could underlie these changes in cortical excitability. Increased input resistance due to a decrease of conductance (presumably to potassium) would cause membrane depolarization and thus increased excitability. A similar explanation has been offered regarding changes of impulse frequency produced during training of the isolated cockroach leg preparation (Woollacott and Hoyle, 1977). Berger and Thompson's finding (Fig. 10) that hippocampal units increase their firing during and following classical conditioning of the rabbit nictitating membrane also might be due to a similar depolarization and decrease of membrane conductance.

These measurements of neural changes with non-gastropod learning models, however, are preliminary. Other causes for these neural changes have not been ruled out. Primary changes in neurons which synaptically affect the cells studied in cockroach, pericruciate cortex, and hippocampal neurons could account for the neural correlates thus far observed with these preparations. As these measurements are extended, however, it will be interesting to compare, across species, their biophysical basis. Even if a precise causal sequence for associative learning cannot be constructed for the more complex nervous systems, the generality of what we learn with *Hermissenda* and other gastropods will be increased by the identification of common neuronal changes.

The neuronal functions of *Hermissenda* are not unique. The voltage-dependent conductances within the soma membrane of the Type B photoreceptor are not unique. Voltage-dependent K^+ and/or Ca^{2+} conductances have been inferred or demonstrated in spinal motor neurons (Blankenship, 1968), frog motor neurons (Barrett and Barrett, 1976), *Helix* neurons (Eckert and Lux, 1976), in vertebrate rods (Fain and Quandt, 1977), barnacle muscle fibers (Hagiwara *et al.,* 1974), molluscan neurons (Connor, 1979; Byrne, 1980), starfish egg (Hagiwara *et al.,* 1974) and hippocampal neurons (Hotson and Prince, 1980). However, the long-term modification of these conductances, as it arises from paired stimulus effects on converging sensory pathways, could be unique to the process of associative learning in a variety of species, all of which have neurons with similar biophysical characteristics.

Biochemical correlates of associative learning

Long-term modification of conductances means long-term membrane changes which must have a biochemical basis. The presence of a slowly decaying light-induced Ca^{2+} current and Ca^{2+}-dependent K^+ current during the long-lasting depolarization of the Type B cell after a light step suggested the possible involvement of cyclic nucleotide metabolism. Recent experiments further suggested some involvement of cyclic nucleotide metabolism in the generation of Type B membrane currents. Intracellular injection of the catalytic subunit of protein kinase (Alkon, Olds, Kuzma, and Neary, in preparation) enhanced the LLD following a light step. Perfusion with IBMX (a phosphodiesterase inhibitor which elevates intracellular cyclic nucleotide levels) also caused prolonged enhancement of the LLD after a brief initial reduction of the LLD. This initial reduction might be due to smaller intracellular cyclic-AMP increments, which were previously found to reduce the light-induced Ca^{++}-current (Alkon, 1979).

Interaction of Ca^{2+} and cyclic nucleotide levels is now thought to occur in a number of tissues (Rasmussen, 1970; Rasmussen *et al.,* 1975; Berridge, 1976; Rasmussen and Goodman, 1975; Malaisse, 1973; Borle, 1974; Lipton *et al.,* 1977; Boron *et al.,* 1978). Intracellular Ca^{2+} could control enzymes which catalyze the synthesis (Rasmussen and Goodman, 1975) or the degradation of cyclic nucleotides. There is also evidence of cyclic nucleotide-dependent phosphorylation of proteins in retinal rods (Farber *et al.*, 1979), Ca^{2+}-dependent protein phosphorylation in neural membranes (Schulman and Greengard, 1978), depolarization-induced protein phosphorylation mediated by Ca^{2+} influx in synaptosomes (Krueger *et al.*, 1977) and light-dependent phosphorylation of rhodopsin (Shichi and Somers, 1978). More recently phosphorylation studies with *Hermissenda* eyes have been conducted in our laboratory. We detected seven phosphoprotein bands in all of the eyes isolated from animals of four groups: trained, unpaired random, and normal controls (Neary *et al.*, 1980). There were significant overall differences between paired and control groups ($P < 0.01$) for bands with approximate molecular weights of 23,000 and 20,000 daltons. The paired group was significantly different from both the random and unpaired control groups ($P < 0.01$) while the control groups were not significantly different from each other. The changes of the two phosphoprotein bands' density (increased 49 and 56% for paired *vs.* random animals) might be related to the depolarization and increased input resistance of the Type B cell following associative training. It will of course be necessary to localize the phosphoprotein changes to photoreceptors within the eye and not neighboring pigment and epithelial cells. We will also attempt to characterize these phosphoproteins and localize them within cellular compartments. The causal sequence for this biochemical change will also be of great interest. How, for instance, might Type B depolarization affect protein kinases and/or phosphatases? Are there direct effects on cyclic-AMP levels, phosphodiesterase activity, etc.? Is protein assembly influenced? Like the biophysical changes, the biochemical changes need not be unique to serve within the context of the neural systems' response to training stimuli, in a manner unique to the process of associative learning. Again, if the biochemical process can be characterized for *Hermissenda* and other gastropods, its generality might be questioned for other species.

Further questions

Photoreception and photoreceptor adaptation are now known to be associated with shifts of intracellular cyclic-GMP (Woodruff and Bownds, 1979) changes of phosphodiesterase activity (Rasmussen, 1970; Rasmussen *et al.*, 1975; Lipton *et al.*, 1977), and protein phosphorylation, as well as changes of intracellular Ca^{2+} concentration (Lisman and Brown, 1972). It could be argued that the biochemical as well as the biophysical changes specific to associatively trained *Hermissenda* involve mechanisms important to photoreception and are therefore less likely to have general significance. As already emphasized, the voltage-dependent conductances which were observed with voltage-clamp of the Type B somata are present in a host of sensory and central neurons in a number of different vertebrate and invertebrate species. Cyclic-nucleotide-Ca^{2+}-protein phosphorylation interaction has also been described for a host of preparations, from synaptosomes to brain slices to retinal rods. These biophysical and biochemical phenomena obviously are available to nervous systems for other functions which are not at all restricted to photoreception. In addition, the behavioral and neural changes specific to associatively trained *Hermissenda* cannot be explained or simulated by differences in light adaptation of the Type B photoreceptors. Light stimuli alone (*i.e.*, in the absence of rotation) produced no behavioral or neural changes (Alkon, 1974; 1975; 1976; Crow and Alkon, 1978; 1980). When animals were exposed to twice the duration of light each day (Lederhendler and Barnes, unpublished observations) their latency to move toward a light source was not reduced (as observed following paired light and rotation). Furthermore, when the photoreceptors are light-adapted during the light phase of the diurnal cycle, the animals approach a light source but do not during the dark phase (Lederhendler *et al.*, 1980). Light adaptation of the photoreceptors, then, is associated with the behavioral effect *opposite* that with associative training. Increasing intensity of light stimuli during training (*i.e.*, increasing the level of light adaptation) cannot approximate the effects of stimulus pairing. Insertion of additional steps of light during training with paired light and rotation does not enhance the behavioral change (Farley and Alkon, in preparation).

Stimulus pairing in essence shifts the Type B membrane potential (by the retrograde spread of the synaptic effects following the stimuli) to more positive levels without bleaching more visual pigment molecules (in this case, rhodopsin) of either Type B or Type A photoreceptors. Increased light intensity will bleach more pigment molecules of both photoreceptor types and thus decrease their response amplitudes to subsequent repeated presentation of the light stimuli during a training period. Cumulative depolarization of the Type B cell produced by light alone would then approach a limit defined by pigment molecule bleaching. This limit of cumulative depolarization could then be exceeded only by an external effect such as the synaptic depolarization which follows stimulus pairing. Light steps of increasing duration ($>$ 1 min) therefore, can be expected and in fact were observed, followed by long-lasting depolarization of the same or decreasing duration (Alkon, unpublished observations). This is also explained by the fact that light-induced Na^+, K^+, and Ca^{2+} conductances have different light intensity-response relationships and adaptation properties (Alkon, 1979). The light-induced K^+ current, for instance, requires brighter lights but shows much less adaptation than do the Na^+ and Ca^{2+} currents (Alkon, 1979). Increasing light duration beyond levels which produce a maximum depolarization during and after a light step can be expected to cause light-induced K^+ currents, larger with respect to the Na^+ and Ca^{2+} currents, that cause more hyperpolarization.

In brief, for the Type B cell, a light-induced Ca^{2+} conductance is enhanced and a voltage-dependent K^+ conductance is reduced by the retrograde spread of synaptic depolarization following stimulus pairing. Stimulus pairing produced this enhanced conductance in a manner which cannot result from light stimuli alone because:

1. The Ca^{2+}, K^+, and Na^+ conductances have different voltage-dependence, and
2. The relative voltage-dependence of these conductances is quite different from their relative light intensity dependence. A stimulus pair is therefore encoded by the synaptic effect on the Type B cell depolarization after light. Similarly, stimulus pair repetition is encoded by the cumulative depolarization which follows associative training.

It is not difficult to imagine that a voltage-dependent synaptic (Magelby and Stevens, 1972) or impulse-generating conductance mediating one sensory stimulus could be enhanced by the synaptic effects caused by pairing with a second stimulus. Stimulus pairing would more likely be encoded by the enhancement of voltage-dependent soma, dendritic, synaptic, and/or axonal conductances in integrating neurons of vertebrate species whose central nervous systems allow a myriad of possible convergence points for associated distinct stimuli within and between sensory modalities. Training with these associated stimuli might then cause a cumulative membrane potential and/or conductance change(s) which, like the Type B depolarization, arise from repeated enhancement of voltage-dependent conductances.

CONCLUSION

The usefulness of gastropod models of associative learning can only be fully assessed in retrospect. Study of these models was undertaken after a number of assumptions, intuitive formulations, and hypotheses were made. It makes intuitive sense that new connections between neurons are not formed during associative learning (Purpura, 1967), because axons and their endings don't grow as quickly as many associations can be learned. It makes intuitive sense that the stimuli associated during learning activate neural pathways which at some point must converge. Presumably, paired stimulation by means of his convergence of pathways produces specific neural signals and neural changes. These in turn cause associative learning behavior. By this line of intuitive reasoning, the convergence point must already exist in order for two stimuli to be associated during learning. If this is true, the number of convergence points will determine the capacity for learning.

The number of convergence points and learning capacity are obviously vastly different for gastropods and humans. Yet if we are true to the intuitive formulations just mentioned there should be a chance, and it is only a chance, for some common underlying physiology. If the gastropod associative learning behavior closely resembles examples of elementary learning (*e.g.* conditioning) of humans, and if primary neural changes mediated by convergence points are identified, the chance of some general significance is there. The more basic our understanding of these primary neural changes, the greater the chance that we can test generality in other species whose neural systems may never be described in their totality.

There are, however, always the caveats that intuition may misguide investigations and models may cloud issues. It may be possible, for instance with a gastropod, to produce a behavioral change which is long-lasting, dependent on paired *vs.* unpaired training and specific to the training stimuli. Yet the mechanism for one animal's "association" of the two training stimuli may be peculiar to this animal and its training experience. As an example, strong shock to a gastropod's head and/or tentacles may stimulate a host of neuronal pathways, none of which converge with the "food" or chemosensory pathway. Instead, strong shock may stimulate the "food" pathway directly, and, when paired with food, produce neural and behavioral changes without the convergence points which we intuitively expect are necessary in a variety of species for associative learning. The mechanism for a neural change produced by the paired effects of shock and food on the same sensory pathway, *i.e.,* without convergence of inputs, seems less likely to have significance for other species in which neuronal convergence is necessary for associative learning.

From our hypotheses about how learning is mediated and limited by convergence points within the nervous system, we would predict that a knowledge of evolutionary constraints on species' learning capacity will help reveal neural mechanisms which may transcend the unique adaptive function of those species. Our gastropod model, then, should be constructed from an understanding of how the animal behaves in and interacts with its natural environment—how it can learn from naturally occurring stimulus patterns. Gastropod models will then less likely be imposed on and more likely be generated and suggested to us by the learning phenomena of interest.

The difficulty of designing the best experiment and choosing the most appropriate model with a minimum of knowledge and helpful intuitions has always confronted scientists. It is a source of reassurance to recall Pavlov's analogous predicament. His productivity as an experimentalist is not in small part, I believe, due to the insight of his intuitive formulations:

"Thus, we may reach the limit of analysis of which a given animal may be capable, *i.e.* most minute natural phenomena may become special stimuli for a definite activity of the organism. We may think that by the same process by which connexions are formed between cortical cells and subcortical centres connexions are also formed between the cortical cells themselves. The excitations produced by phenomena taking place simultaneously in the outside world are thus complex. These complex excitations may become, under corresponding conditions, conditioned stimuli, and be differentiated by means of the just-indicated process of inhibition from other closely related complex stimuli" (Pavlov, 1927).

ACKNOWLEDGMENTS

I wish to acknowledge the invaluable assistance of Mrs. Jeanne Kuzirian in all aspects of this article's preparation. I am also grateful to Ms. Linda and Mr. Bob Golder for their gifted and dedicated help in preparing the illustrations.

LITERATURE CITED

ADEY, W. R., C. W. DUNLOP, AND C. E. HENDRIK, 1960. Hippocampal slow waves: distribution and phase relationships in the course of approach learning. *Arch. Neurol.,* **3**: 74–90.

AGERSBORG, H. P. K., 1922. Some observations on qualitative chemical and physical stimulations in nudibranchiate mollusks with special reference to the role of the rhinophore. *J. Exp. Zool.,* **36**: 423–444.

AGERSBORG, H. P. K., 1925. The sensory receptors and the structure of the oral tentacles of of the nudibranchiate mollusk *Hermissenda crassicornis* syn. Hermissenda opalescens. *Acta Zool. (Stockholm)*, **6**: 167–182.

AKAIKE, T., AND D. L. ALKON, 1980. Sensory convergence on central visual neurons in *Hermissenda. J. Neurophysiol.,* **44**: 501–513.

ALKON, D. L., 1973a. Intersensory interactions in *Hermissenda. J. Gen. Physiol.,* **62**: 185–202.

ALKON, D. L., 1973b. Neural organization of a molluscan visual system. *J. Gen. Physiol.,* **61**: 444–461.

ALKON, D. L., 1974a. Associative training of *Hermissenda. J. Gen. Physiol.,* **64**: 70–84.

ALKON, D. L., 1974b. Sensory interactions in the nudibranch mollusc *Hermissenda crassicornis. Fed. Proc.,* **33**: 1083–1090.

ALKON, D. L., 1975. Neural correlates of associative training in *Hermissenda. J. Gen. Physiol.,* **65**: 46–56.

ALKON, D. L., 1976a. The economy of photoreceptor function in a primitive nervous system. Pp. 410–426 in F. Zettler and R. Weiler, Eds., *Neural Principles in Vision,* Springer Verlag, New York.

ALKON, D. L., 1976b. Neural modification by paired sensory stimuli. *J. Gen. Physiol.,* **68**: 341–358.

ALKON, D. L., 1979. Voltage-dependent calcium and potassium ion conductances: A contingency mechanism for an associative learning model. *Science,* **205**: 810–816.

ALKON, D. L., 1980. Membrane depolarization accumulates during acquisition of an associative behavioral change. *Science,* **209**: in press.

ALKON, D. L., T. AKAIKE, AND J. F. HARRIGAN, 1978. Interaction of chemosensory, visual and statocyst pathways in *Hermissenda. J. Gen. Physiol.,* **71**: 177–194.

ALKON, D. L., AND A. BAK, 1973. Hair cell generator potentials. *J. Gen. Physiol.,* **61**: 619–637.

ALKON, D. L., AND M. G. F. FUORTES, 1972. Responses of photoreceptors in *Hermissenda. J. Gen. Physiol.,* **60**: 631–649.

Alkon, D. L., and Y. Grossman, 1978a. Evidence for non-synaptic neuronal interaction. *J. Neurophysiol.*, **41**: 640–653.

Alkon, D. L., and Y. Grossman, 1978b. Long-lasting depolarization and hyperpolarization in eye of *Hermissenda*. *J. Neurophysiol.*, **41**: 1328–1342.

Altman, J. A., and N. M. Tyrer, 1977. The locust wing hinge stretch receptors. II. Variation, alternative pathways and "mistakes" in the central arborizations. *J. Comp. Neur.*, **172**: 431–440.

Aranda, L. C., and J. V. Luco, 1969. Further studies on an electrical correlate to learning: experiments in an isolated insect ganglion. *Physiol. Behav.*, **4**: 133–137.

Barrett, E., and J. Barrett, 1976. Separation of two voltage-sensitive potassium currents, and demonstration of a tetrodotoxin-resistant calcium current in frog motorneurones. *J. Physiol., (Lond.)*, **255**: 737–774.

Baudry, M., and G. Lynch, 1980. Regulation of hippocampal glutamate receptors: Evidence for the involvement of a calcium-activated protease. *Proc. Natl. Acad. Sci. U.S.A.*, **77**: 2298–2302.

Baudry, M., and G. Lynch, 1980. Hypothesis regarding the cellular mechanisms responsible for long-term synaptic potentiation in the hippocampus. *Exp. Neurol.*, **68**: 202–204.

Berger, T. W., and R. F. Thompson, 1978a. Identification of pyramidal cells as the critical elements in hippocampal neuronal plasticity during learning. *Proc. Nat. Acad. Sci. U.S.A.*, **75**: 1572–1576.

Berger, T. W., and R. F. Thomson, 1978b. Neuronal plasticity in the limbic system during classical conditioning of the rabbit nictitating membrane response. I. The Hippocampi. *Brain Res.*, **145**: 323–346.

Berger, T. W., and R. F. Thompson, 1978c. Neuronal plasticity in the limbic system during classical conditioning of the rabbit nictitating membrane response. II. Septum and mammillary bodies. *Brain Res.*, **156**: 293–314.

Berridge, M. J., 1976. Interaction of cyclic nucleotides and calcium in the control of cellular activity. *Adv. Cyclic Nucleotide Res.*, **6**: 1–98.

Blakemore, C., J. Nachimias, and P. Sutton, 1970. The perceived spatial frequency shift: evidence for frequency-selective neurons in the human brain. *J. Physiol. (Lond.)*, **210**: 727–750.

Blankenship, J. E., 1968. Action of tetrodotoxin on spinal motoneurons of the cat. *J. Neurophysiol.*, **31**: 186–194.

Bodian, D., 1936. A new method for staining nerve fibers and nerve endings in mounted paraffin sections. *Anat. Rec.*, **65**: 89–97.

Borle, A. B., 1974. Cyclic AMP stimulation of calcium efflux from kidney, liver and heart mitochondria. *J. Membr. Biol.*, **16**: 221–236.

Boron, W., J. Russell, M. Brodwick, D. Keifer, and A. Roos, 1978. Influence of cyclic AMP on intracellular pH regulation and chloride fluxes in barnacle muscle fibers. *Nature*, **276**: 511–513.

Bruner, J., and D. Kennedy, 1970. Habituation: occurrence at a neuromuscular junction. *Science*, **169**: 92–94.

Bruner, J., and L. Tauc, 1964. Les modifications de l'activité synaptique au cours de l'habituation chez l'*Aplysie*. *J. Physiol. (Paris)*, **56**: 306–307.

Bruner, J., and L. Tauc, 1965. La plasticité synaptique impliquée dans le processus d'habituation chez l'*Aplysie*. *J. Physiol. (Paris)*, **57**: 230–231.

Bruner, J., and L. Tauc, 1966a. Habituation at the synaptic level in *Aplysia*. *Nature*, **210**: 37–39.

Bruner, J., and L. Tauc, 1966b. Long-lasting phenomena in the molluskan nervous system. *Symp. Soc. for Exp. Biol.*, **20**: 457–475.

Buchwald, J. S., E. S. Halas, and S. Schramm, 1966. Changes in cortical and subcortical unit activity during conditioning in chronic cats. *Physiol. Behav.*, **I**: 11–22.

Bullock, T. H., and G. C. Quarton, 1966. Simple systems for the study of learning mechanisms. *Neurosci. Res. Prog. Bull.*, **4**: 203–327.

Bures, J., and O. Buresova, 1967. Plastic changes of unit activity based on reinforcing properties of extracellular stimulation of single neurons. *J. Neurophysiol.*, **30**: 98–113.

Burrows, M., 1973. Physiological and morphological properties of the metathoracic common inhibitory neuron of the locust. *J. Comp. Physiol.*, **82**: 59–78.

Burrows, M., and G. A. Horridge, 1974. The organization of inputs to motoneurons of the locust metathoracic leg. *Philos. Trans. R. Soc. Lond. B. Biol.*, **269**: 49–94.

Burrows, M., and G. Hoyle, 1973. Neural mechanisms underlying behavior in the locust *Schistocera gregaria*. III. Topography of limb motoneurons in the metathoracic ganglion. *J. Neurobiol.,* **4**: 167–186.

Byrne, J. H., 1980a. Analysis of ionic conductance mechanisms in motor cells mediating inking behavior in *Aplysia californica*. *J. Neurophysiol.,* **43**: 630–650.

Byrne, J. H., 1980b. Quantitative aspects of ionic conductance mechanisms contributing to firing pattern of motor cells mediating inking behavior in *Aplysia californica*. *J. Neurophysiol.,* **43**: 651–668.

Carew, T. J., and E. R. Kandel, 1973. Acquisition and retention of long-term habituation in *Aplysia:* correlation of behavioral and cellular process. *Science,* **182**: 1158–1160.

Carew, T. J., H. M. Pinsker, and E. R. Kandel, 1972. Long-term habituation of a defensive withdrawal reflex in *Aplysia*. *Science,* **175**: 451–454.

Carew, T. J., V. F. Castellucci, and E. R. Kandel, 1971. An analysis of dishabituation and sensitization of the gill-withdrawal reflex in *Aplysia*. *Int. J. Neurosci.,* **2**: 79–98.

Castellucci, V. F., H. M. Pinsker, I. Kupferman, and E. R. Kandel, 1970. Neuronal mechanisms of habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*. *Science,* **167**: 1745–1748.

Cohen, D. H., 1969. Development of a vertebrate experimental model for cellular neurophysiologic studies of learning. *Conditional Reflex,* **4**: 61–80.

Cohen, D. H., and R. L. Macdonald, 1976. Involvement of the avian hypothalamus in defensively conditioned heart rate change. *J. Comp. Neurol.,* **167**: 465–480.

Cohen, M. J., and J. W. Jacklet, 1967. The functional organization of motor neurons in an insect ganglion. *Philos. Trans. R. Soc. Lond. B. Biol.,* **252**: 563–571.

Connor, J. A., 1979. Calcium current in molluscan neurones: measurement under conditions which maximize its visibility. *J. Physiol. (Lond.),* **286**: 41–60.

Connor, J. A., and C. F. Stevens, 1971. Voltage-clamp studies of a transient outward membrane current in gastropod neural somata. *J. Physiol. (Lond.),* **213**: 21–30.

Crow, T. J., and D. L. Alkon, 1978a. Neural correlates of an associative behavioral change in *Hermissenda crassicornis*. *Soc. Neurosci.,* **4**: 191.

Crow, T. J., and D. L. Alkon, 1978b. Retention of an associative behavioral change in *Hermissenda*. *Science,* **201**: 1239–1241.

Crow, T. J., and D. L. Alkon, 1980a. Associative behavioral modification in *Hermissenda*: cellular correlates. *Science,* **209**: 412–414.

Crow, T., and D. L. Alkon, 1980b. Neural correlates of a long-term associative behavioral modification in *Hermissenda crassicornis*. *Fed. Proc.* **39**: 596.

Crow, T., E. Heldman, V. Hacopian, R. Enos, and D. L. Alkon, 1979. Ultrastructure of photoreceptors in the eye of *Hermissenda* labeled with intracellular injections of horseradish peroxidase. *J. Neurocytol.* **8**: 181–195.

Crow, T., and N. Offenbach, 1979. Response specificity following behavioral training in the nudibranch mollusk *Hermissenda crassicornis*. *Biol. Bull.,* **157**: 364.

Davis, W. J., G. J. Mpitsos, and J. M. Pinneo, 1974. The behavioral hierarchy of the mollusk *Pleurobranchaea*. I. The dominant position of feeding behavior. *J. Comp. Physiol.* **90**: 207–224.

Davis, W. J., J. Villet, D. Lee, M. Rigler, R. Gillette, and E. Prince, 1980. Selective and differential avoidance learning in the feeding and withdrawal behavior of *Pleurobranchaea californica*. *J. Comp. Physiol.,* **138**: 157–165.

DeFelice, L. J., and D. L. Alkon, 1977. Voltage noise from hair cells during mechanical stimulation. *Nature,* **269**: 613–615.

Detwiler, P. B., and D. L. Alkon, 1973. Hair cell interactions in the statocyst of *Hermissenda*. *J. Gen. Physiol.,* **62**: 618–642.

Disterhoft, J. F., J. Nurnberger, and W. C. Corning, 1968. "P-R" differences in intact cockroaches as a function of testing interval. *Psychonomic Science, Section on Animal and Physiological Psychology,* **12**: 205–206.

Disterhoft, J. F., R. Haggerty, and W. C. Corning, 1971. An analysis of leg position learning in the cockroach yoked control. *Physiol. Behav.,* **7**: 359–362.

Doty, R. W., 1961. The role of subcortical structures in conditioned reflexes. *Ann. N.Y. Acad. Sci.,* **92**: 939–945.

Dowing, J. E., and B. B. Boycott, 1965. Neural connections of the retina: fine structure of the inner plexiform layer. *Cold Spr. Harb. Symp. Quant. Biol.,* **30**: 393–402.

Dunwiddie, T. V., and G. Lynch, 1979. The relationship between extracellular calcium concentration and the induction of hippocampal long-term potentiation. *Brain Res.*, **169**: 103–110.

Eckert, R., and H. D. Lux, 1976. A voltage-sensitive persistent calcium conductance in neuronal somata of *Helix*. *J. Physiol. (Lond.)* **254**: 129–151.

Eisenstein, E. G., 1968. Assessing the influence of pharmacological agents on shock avoidance learning in simpler systems. *Brain Res.* **11**: 471–480.

Eisenstein, E. M., 1970a. A comparison of *activity* and *position* response measures of avoidance learning in the cockroach, *P. americana*. *Brain Res.*, **21**: 143–147.

Eisenstein, E. M., 1970b. The retention of shock avoidance learning in the cockroach, *P. americana*. *Brain Res.* **21**: 148–150.

Eisenstein, E. M., and M. J. Cohen, 1965. Learning in an isolated prothoracic insect ganglion. *Anim. Behav.* **13**: 104–108.

Eisenstein, E. M., G. Kemeny, I. D. Skurnick, and J. T. Harris, 1972. Transfer of information within the cockroach central nervous system during shock avoidance learning: is there a nerve impulse code(s) for learned information? *Int. J. Neurosci.*, **3**: 93–98.

Eisenstein, E. M., and G. H. Krasilovsky, 1967. Studies of learning in isolated insect ganglia. Pp. 329–332 in C.A.G. Wiersma, Ed., *Invertebrate Nervous Systems*, Chicago. The University of Chicago Press.

Eisenstein, E. M., and B. Peretz, 1973. Comparative aspects of habituation in invertebrates. Pp. 1–34 in H. Peeke and M. J. Herz, Eds., *Habituation*, Vol. 2, *Electrophysiological Substrates.*

Epstein, R., and L. Tauc, 1970. Heterosynaptic facilitation and posttetanic potentiation in *Aplysia* nervous system. *J. Physiol.*, **209**: 1–23.

Evarts, E. V., 1973. Motor cortex reflexes associated with learned movement. *Science*, **179**: 501–503.

Fain, G. L., and F. N. Quandt, 1977. Calcium-dependent regenerative responses in rods. *Nature*, **269**: 707–709.

Farber, D. B., M. B. Brown, and R. N. Lolley, 1979. Cyclic nucleotide dependent protein kinase and the phosphorylation of endogenous proteins of retinal rod outer segments. *Biochemistry*, **18**: 370–378.

Farley, J., and D. L. Alkon, 1980. Neural organization predicts stimulus specificity for a retained associative behavioral change. *Soc. Neurosci.*, **6**: 786.

Fetz, E. E., and D. B. Finocchio, 1971. Operant conditioning of specific patterns of neural and muscular activity. *Science*, **174**: 431–435.

Fredman, S. M., and B. Jahan-Parwar, 1980. Processing of chemosensory and mechanosensory information in identifiable *Aplysia* neurons. *Comp. Biochem. Physiol.*, **66**A: 25–34.

Fuster, J. M., and G. E. Alexander, 1971. Neuron activity related to short-term memory. *Science*, **173**: 652–654.

Gelperin, A., 1975. Rapid food-aversion learning by a terrestrial mollusk. *Science*, **189**: 567–570.

Getchell, T. V., 1977. Analysis of intracellular recordings from salamander olfactory epithelium. *Brain Res.*, **123**: 275–286.

Gillette, R., and W. J. Davis, 1977. The role of the metacerebral giant neuron in the feeding behavior of *Pleurobranchaea*. *J. Comp. Physiol.*, **116**: 129–159.

Gillette, R., M. V. Gillette, and W. J. Davis, 1980. Action-potential broadening and endogenously sustained bursting are substrates of command ability in a feeding neuron of *Pleurobranchaea*. *J. Neurophysiol.*, **43**: 669–685.

Goodman, C. S., 1978. Isogenic grasshoppers: genetic variability in the morphology of identified neurons. *J. Comp. Neurol.*, **182**: 681–706.

Gormezano, I., and J. W. Moore, 1969. History and method. Pp. 121–203 in M. M. Marx, Ed., *Learning Processes*, Macmillan, Toronto.

Gormezano, I., N. Schneiderman, E. Deaux, and I. Fuentes, 1962. Nictitating membrane: classical conditioning and extinction in the albino rabbit. *Science* **138**: 33–34.

Grossman, Y., D. L. Alkon, and E. Heldman, 1979. A common origin of voltage noise and generator potentials in statocyst hair cells. *J. Gen. Physiol.*, **73**: 23–48.

Hagiwara, S., J. Fukuda, and D. C. Eaton, 1974. Membrane currents carried by Ca, Sr, and Ba in barnacle muscle fiber during voltage-clamp. *J. Gen. Physiol.*, **63**: 564–578.

HARRIS, J. D., 1943. Habituatory response decrement in the intact organism. *Psychol. Bull.,* **40**: 385–422.

HELDMAN, E. Y. GROSSMAN, T. P. JERUSSI, AND D. L. ALKON, 1979. Cholinergic features of photoreceptor synapses in *Hermissenda. J. Neurophysiol.,* **42**: 153–165.

HIRSCH, H. V. B., AND D. N. SPINELLI, 1970. Visual experience modifies distribution of horizontally and vertically oriented receptive fields in cats. *Science,* **168**: 769–871.

HIRSCH, H. V. B., AND D. N. SPINELLI, 1971. Modification of the distribution of receptive field orientation during development. *Exp. Brain Res.,* **13**: 509–527.

HORRIDGE, G. A., 1959. Analysis of the rapid responses of *Nereis* and *Harmothoe* (Annelida). *Proc. R. Soc. Lond. B Biol.,* **150**: 245–262.

HORRIDGE, G. A., 1962a. Learning of leg position by the ventral nerve cord in headless insects. *Proc. R. Soc. Lond. B Biol.,* **157**: 33–52.

HORRIDGE, G. A., 1962b. Learning of leg position by headless insects. *Nature,* **193**: 697–698.

HORRIDGE, G. A., 1965. The electrophysiological approach to learning in isolated ganglia. *Anim. Behav.,* **12**, 163–182.

HOTSON, J. R., AND D. A. PRINCE, 1980. A calcium-activated hyperpolarization follows repetitive firing in hippocampal neurons. *J. Neurophysiol.,* **43**: 409–419.

HOYLE, G., 1964. Exploration of neuronal mechanisms underlying behavior in insects. Pp. 346–376 in R. F. Reiss, Ed., *Neural Theory and Modeling,* Stanford University Press, Stanford.

HOYLE, G., 1965. Neurophysiological studies of "learning" in headless insects. Pp. 203–232 in J. E. Treherne and J. W. L. Beament, Eds., *The Physiology of the Insect Central Nervous System,* Academic Press, New York.

HOYLE, G., 1970. Cellular mechanisms underlying behavior-neuroethology. *Advances in Insect Physiology* **7**: 349–444.

HOYLE, G., AND M. BURROWS, 1973a. Neural mechanisms underlying behavior in the locust *Shistocerca gregaria.* I. Physiology of identified motoneurons in the metathoracic ganglion. *J. Neurobiol.* **4**: 3–41.

HOYLE, G., AND M. BURROWS, 1973b. Neural mechanisms underlying behavior in the locust *Shistocerca gregaria.* II. Integrative activity in metathoracic neurons. *J. Neurobiol.,* **4**: 43–67.

HUBEL, D. H., AND T. N. WIESEL, 1970. The period of susceptibility to the physiological effects of unilateral eye closure in kittens. *J. Physiol.,* **206**: 419–436.

HUGHES, G. M., AND L. TAUC, 1963. An electrophysiological study of the anatomical relations of two giant nerve cells in *Aplysia depilans. J. Exp. Biol.,* **40**: 469–486.

HUMPHREY, G., 1930. Le Chatelier's role, and the problem of habituation and dehabituation in *Helix albolabris. Psychol. Forsch.,* **13**: 113–127.

JACKLET, J. W., 1977. Neuronal circadian rhythm: phase shifting by a protein synthesis inhibitor. *Science,* **198**: 69–71.

JACKLET, J. W., AND J. RINE, 1977. Facilitation at neuromuscular junctions: contribution to habituation and dishabituation of the *Aplysia* gill withdrawal reflex. *Proc. Natl. Acad. Sci. U.S.A.,* **74**: 1267–1271.

JASPER, H. H., G. RICCI, AND B. DOANE, 1960. Microelectrode analysis of cortical cell discharge during avoidance conditioning in the monkey. *EEG Clin. Neurophysiol. (Suppl.),* **13**: 137–155.

JENNINGS, K. R., L. K. KACZMAREK, AND F. STRUMWASSER, 1979. Protein phosphorylation during afterdischarge of the neuroendocrine bag cells in *Aplysia. Soc. Neurosci.,* **5**: 249.

JERUSSI, T. P., AND D. L. ALKON, 1980. Light responses of motor units in a marine mollusc. *Fed. Proc.* **39**: 596.

JOHN, E. R., 1967. Electrophysiological studies of conditioning. Pp. 690–704, in G. C. Quarton, T. Melnechuk, and F. O. Schmitt, Eds., *The Neurosciences: A Study Program,* Rockefeller University Press, New York.

JOHN, E. R., F. BARTLETT, M. SHIMOKOCHI, AND D. KLEINMAN, 1973. Neural readout from memory. *J. Neurophysiol.,* **36**: 893–924.

JOHN, E. R., AND K. F. KILLAM, 1959. Electrophysiological correlates of avoidance conditioning in the cat. *J. Pharmacol. Exper. Ther.,* **125**: 252–274.

KACZMAREK, L. K., K. R. JENNINGS, AND F. STRUMWASSER, 1978. Neurotransmitter modulation of phosphodiesterase inhibitor effects and cyclic AMP correlates of afterdischarge in peptidergic neurites. *Proc. Natl. Acad. Sci. U.S.A.*, **75**: 5200–5204.

Kaczmarek, L. K., K. R. Jennings, and F. Strumwasser, 1979. Cyclic AMP analog generates afterdischarge in *Aplysia* bag cell neurons. *Soc. Neurosci.*, **5**: 249.

Kandel, E. R., and L. Tauc, 1964. Mechanisms of prolonged heterosynaptic facilitation. *Nature* **202**: 145–147.

Kandel, E. R., and I. Tauc, 1965a. Heterosynaptic facilitation in neurons of the abdominal ganglion of *Aplysia depilans*. *J. Physiol.*, **181**: 1–27.

Kandel, E. R., and L. Tauc, 1965b. Mechanisms of heterosynaptic facilitation in the giant cell of the abdominal ganglion of *Aplysia depilans*. *J. Physiol.*, **181**: 28–47.

Kater, S. B., 1974. Feeding in *Helisoma trivolvis*: the morphological and physiological basis of a fixed action pattern. *Am. Zool.*, **14**: 1017–1036.

Keene, J. J., 1973. Reward-associated inhibition and pain-associated excitation lasting seconds in single intralaminar thalamic units. *Brain Res.*, **64**: 211–224.

Krasne, F. B., and K. S. Woodsmall, 1969. Waning of the crayfish escape response as a result of repeated stimulation. *Anim. Behav.*, **17**: 416–424.

Krueger, B. K., J. Forn, and P. Greengard, 1977. Depolarization-induced phosphorylation of specific proteins, mediated by calcium ion influx, in rat brain synaptosomes. *J. Biol. Chem.*, **252**: 2764–2773.

Kupferman, I., V. Castellucci, H. Pinsker, and E. R. Kandel, 1970. Neuronal correlates of habituation and dishabituation of the gill-withdrawal reflex of *Aplysia*. *Science*, **167**: 1743–1745.

Lasansky, A., 1971. Synaptic organization of cone cells in the turtle retina. *Phil. Trans. R. Soc. Lond. B Biol.*, **252**: 265–281.

Lederhendler, I. I., E. S. Barnes, and D. L. Alkon, 1980. Complex responses to light of the nudibranch *Hermissenda crassicornis*. *Behav. Neural Biol.*, **28**: 218–230.

Lee, R. M., and R. J. Liegeois, 1974. Motor and sensory mechanisms of feeding in *Pleurobranchaea*. *J. Neurobiol.*, **5**: 545–564.

Lipton, S. A., H. Rasmussen, and J. D. Dowling, 1977. Electrical and adaptive properties of rod photoreceptors in *Bufo marinus*. *J. Gen. Physiol.*, **70**: 771–791.

Lisman, J. E., and J. E. Brown, 1972. The effects of intracellular iontophoretic injection of calcium and sodium ions on the light responses of Limulus ventral photoreceptors. *J. Gen. Physiol.*, **59**: 701–719.

Livingston, R. B., 1966. Brain mechanisms in conditioning and learning. *Neurosci. Res. Prog. Bull.*, **4**: 349–354.

Llinas, R., R. Baker, and W. Prect, 1972. Blockage of inhibition by ammonium acetate action on chloride pump in cat trochlear motorneurons. *J. Neurophysiol.* **37**: 522–532.

Llinas, R., and T. Hess, 1976. Tetrodotoxin-resistant dendridic spikes in avian Purkinje cells. *Proc. Natl. Acad. Sci. U.S.A.* **73**: 2520–2523.

Lubbock, J., 1958. On the digestive and nervous systems of *Coccus hesperidum*. *Proc. R. Soc.*, **9**: 480–486.

Lukowiak, K., 1979. Food increases CNS suppressive control over gill reflex behaviors in *Aplysia*. *Soc. Neurosci.*, **5**: 252.

Lukowiak, K., and J. W. Jacklet, 1972. Habituation and dishabituation: interactions between peripheral and central nervous systems in *Aplysia*. *Science*, **178**: 1306–1308.

Lukowiak, K., and J. Jacklet, 1975. Habituation and dishabituation mediated by the peripheral and central neural circuits of the siphon of *Aplysia*. *J. Neurobiol.*, **6**: 103–200.

Macagno, E. R., V. Lopresti, and C. Levinthal, 1973. Structure and development of neuronal connections in isogenic organisms: variations and similarities in the optic system of *Daphnia magna*. *Proc. Nat. Acad. Sci. U.S.A.*, **70**: 57–61.

Magelby, K. L., and C. F. Stevens, 1972. A quantitative description of end-plate currents. *J. Physiol.* **223**: 173–197.

Malaisse, W. J., 1973. The stimulus secretion coupling of glucose induced insulin release. Part 12. Effects of diazoxide and gliclazide upon calcium-45 efflux from perfused islets. *Diabetologica*, **9**: 1–5.

Miller, N. E., 1967. Certain facts of learning relevant for its physical basis. Pp. 643–652 in G. C. Quarton, T. Melnechuk, F. O. Schmitt, Eds., *The Neurosciences: A Study Program*, Vol. 2. New York, Rockefeller University Press.

Mpitsos, G. J., and S. D. Collins, 1975. Learning: rapid aversive conditioning in the gastropod mollusk *Pleurobranchaea*. *Science*, **188**: 954–956.

Neary, J. T., T. J. Crow, and D. L. Alkon, 1980. Biochemical correlates of a long-term associative behavioral modification in *Hermissenda*: changes in the levels of specific phosphoprotein bands following a conditioning procedure. *Soc. Neurosci.*, **6**: 591.

Neher, E., and C. F. Stevens, 1977. Conductance fluctuations and ionic pores in membranes. *Ann. Rev. Biophys. Bioeng.*, **6**: 345–381.

Nienhuis, R., and J. Olds, 1978. Changes in unit responses to tones after food reinforcement in the auditory pathway of the rat: intertrial arousal. *Exp. Neurol.*, **59**: 229–242.

Norman, R. J., J. S. Buchwald, and J. R. Villablanca, 1977. Classical conditioning with auditory discrimination of the eye blink in decerebrate cats. *Science*, **196**: 551–553.

Olds, J., J. F. Disterhoft, M. Segal, C. L. Kornblith, and R. Hirsch, 1972. Learning centers of rat brain mapped by measuring latencies of conditional unit responses. *J. Neurophysiol.*, **35**: 202–219.

Olds, J., and T. Hiramo, 1969. Conditional responses of hippocampal and other neurons. *EEG Clin. Neurophysiol.* **26**: 159–166.

Pavlov, I. P. S. P., 1927. *Conditioned Reflexes.* Translated by G. V. Anrep. Oxford University Press, London.

Pearson, K. G., 1972. Central programming and reflex control of walking in the cockroach. *J. Exp. Biol.*, **56**: 173–193.

Pearson, K. G., and C. S. Goodman, 1979. Correlation of variability in structure with variability in synaptic connections of an identified interneuron in locusts. *J. Comp. Neurol.*, **184**: 141–166.

Peretz, B., 1969. Central neuron initiation of periodic gill movements. *Science*, **116**: 1167–1172.

Peretz, B., 1970. Habituation and dishabituation in the absence of a central nervous system. *Science*, **169**: 373–381.

Peretz, B., and J. Estes, 1974. Histology and histochemistry of the peripheral neural plexus in the *Aplysia* gill. *J. Neurobiol.* **5**: 3–19.

Peretz, B., and D. B. Howieson, 1973. Central influence on peripherally mediated habituation of an *Aplysia* gill withdrawal response. *J. Comp. Physiol.*, **84**: 1–18.

Peretz, B., J. W. Jacklet, and K. Lukowiak, 1976. Habituation of reflexes in *Aplysia*: Contribution of the peripheral and central nervous systems. *Science*, **191**: 396–399.

Peretz, B., and R. Moller, 1974. Control of habituation of the withdrawal reflex by the gill ganglion in *Aplysia*. *J. Neurobiol.*, **5**: 191–212.

Pitman, R. M., C. D. Tweedle, and M. J. Cohen, 1973. The form of nerve cells: determination by cobalt impregnation. Pp. 83–97 in S. B. Kater and C. Nicholson, Eds., *Intracellular Staining in Neurobiology.* Springer-Verlag, New York.

Pribram, K. H., D. N. Spinelli, and M. C. Kornback, 1967. Electrocortical correlates of stimulus response and reinforcement. *Science*, **157**: 94–96.

Pritchatt, D., 1968. Avoidance of electric shock by the cockroach *Periplaneta americana*. *Anim. Behav.*, **16**: 178–185.

Purpura, D. P., 1967. Comparative physiology of dendrites. Pp. 372–393 in G. C. Quarton, T. Melnechuk, and F. O. Schmitt, Eds., *The Neurosciences: A Study Program.* Rockefeller University Press, New York.

Rasmussen, H., 1970. Cell communication, calcium ion, and cyclic adenosine monophosphate. *Science*, **170**: 404–412.

Rasmussen, H., and D. B. P. Goodman, 1975. Calcium and cAMP as interrelated intracellular messengers. *Ann. N.Y. Acad. Sci.*, **253**: 789–796.

Rasmussen, H., P. Jensen, W. Lake, N. Friedman, and D. B. P. Goodman, 1975. Cyclic nucleotides and cellular calcium metabolism. *Adv. Cyclic Nucleotide Res.* **5**: 375–394.

Reingold, S. C., and A. Gelperin, 1979. Feeding modulation in *Limax*: behavior and physiology. *Soc. Neurosci.*, **5**: 259.

Rescorla, R. A., 1967. Pavlovian conditioning and its proper control procedures. *Psychol. Rev.*, **74**: 71–80.

Runion, H. I., and P. N. R. Usherwood, 1968. Tarsal receptors and leg reflexes in the locust and grasshopper. *J. Exp. Biol.*, **49**: 421–436.

Schulman, H., and P. Greengard, 1978. Ca^{2+}-dependent protein phosphorylation system in membranes from various tissues, and its activation by "calcium-dependent regulator." *Proc. Nat. Acad. Sci. U.S.A.*, **75**: 5432–5436.

SHEPHERD, G., 1979. *The Synaptic Organization of the Brain.* Oxford University Press, New York. 436p.

SHICHI, H., AND R. L. SOMERS, 1978. Light-dependent phosphorylation of rhodopsin. *J. Biol. Chem.,* **253**: 7040.

SHOUKIMAS, J. J., AND D. L. ALKON, 1980. Voltage-dependent, early outward current in a photoreceptor of *Hermissenda crassicornis. Soc. Neurosci.,* **6**: 17.

SPRAY, D. C., M. E. SPIRA, AND M. V. L. BENNETT, 1980. Peripheral fields and branching patterns of buccal mechanosensory neurons in the opisthobranch mollusc, *Navanax inermis. Brain Res.,* **182**: 253–270.

STENSAAS, L. J., S. S. STENSAAS, AND O. TRUJILLO-CENOZ, 1969. Some morphological aspects of the visual system of *Hermissenda crassicornis* (Mollusca: Nudibranchia). *J. Ultrastruct. Res.,* **27**: 510.

STINNAKRE, J., AND L. TAUC, 1969. Central neuronal response to the activation of osmoreceptors in the osphradium of *Aplysia. J. Exp. Biol.* **51**: 347–361.

STOMMEL, E. W., R. E. STEPHENS, AND D. L. ALKON, 1980. Motile statocyst cilia transmit rather than transduce mechanical stimuli. *J. Cell. Biol.,* **84**: in press.

STRETTON, A. O. W., AND E. KRAVITZ, 1973. Intracellular dye injection: the selection of Procion yellow and its application in preliminary studies of neuronal geometry of the lobster nervous system. Pp. 21–40 in S. B. Kater and C. Nicholson, Eds., *Intracellular Staining in Neurobiology.* Springer-Verlag, New York.

STRUMWASSER, F., 1965. The demonstration and manipulation of a circadian rhythm in a single neuron. Pp. 442–462 in J. A. Schoff, Ed., *Circadian Clocks.* North Holland Publishing Co., Amsterdam.

STRUMWASSER, F., J. W. JACKLET, AND R. B. ALVAREZ, 1969. A seasonal rhythm in the neural extract induction of behavioral egg-laying in *Aplysia. Comp. Biochem. Physiol.* **29**: 197–206.

TABATA, M., AND D. L. ALKON, 1979. Control of synaptic feedback in a molluscan visual system. *Invest. Ophthalmol. Vis. Sci., Suppl.*: 77.

TAUC, L., AND R. EPSTEIN, 1967. Heterosynaptic facilitation as a distinct mechanism in *Aplysia. Nature,* **214**: 724–725.

THOMPSON, E. B., J. H. SCHWARTZ, AND E. R. KANDEL, 1976. A radioautographic analysis in the light and electron microscope of identified *Aplysia* neurons and their processes after intrasomatic injection of L{^{3}H} fucose. *Brain Res.,* **112**: 251–281.

THOMPSON, R. F., AND W. A. SPENCER, 1966. Habituation: A model phenomenon for the study of neuronal substrates of behavior. *Psychol. Rev.,* **73**: 16–43.

THOMPSON, W. F., AND G. S. STENT, 1976. Neuronal control of heartbeat in the medicinal leech. II. Intersegmental coordination of heart motor neuron activity by heart interneurons. *J. Comp. Physiol.,* **111**: 281–307.

THORPE, W. H., 1963. *Learning and Instinct in Animals.* Harvard University Press, Cambridge, MA.

TOLEDO-MORRELL, L., T. J. HOEPPNER, AND F. MORRELL, 1979. Conditioned inhibition: Selective response of single units. *Science,* **204**: 528–530.

VORONIN, L. L., 1971. Microelectrode investigations of cellular analogs of learning. *Sov. Neurol. Psych.,* **4**: 99–125.

VON BAUMGARTEN, R., 1970. Plasticity in the nervous system at the unitary level. Pp. 260–271 in F. O. Schmitt, Ed., *The Neuroscience: Second Study Program.* Rockefeller University Press, New York.

VON BAUMGARTEN, R., AND B. DJAHANPARWAR, 1967. Time course of repetitive heterosynaptic facilitation in *Aplysia californica. Brain Res.,* **4**: 295–297.

VON BAUMGARTEN, R., AND T. HUKUHARA, 1969. The role of interstimulus interval in heterosynaptic facilitation in *Aplysia californica. Brain Res.,* **16**: 369–381.

WALTERS, E. T., T. J. CAREW, AND E. R. KANDEL, 1979. Classical conditionng in *Aplysia californica. Proc. Nat. Acad. Sci. U.S.A.,* **76**: 6675–6679.

WEIGHT, F. F., 1974. Physiological mechanisms of synaptic modulation. Pp. 929–941 in F. O. Schmitt and F. G. Worden, Eds., *The Neurosciences: Third Study Program.* MIT Press, Cambridge, Mass.

WIESEL, T. N., AND D. H. HUBEL, 1965. Comparison of the effects of unilateral and bilateral eye closure on cortical unit responses in kittens. *J. Neurophysiol.,* **28**: 1029–1040.

Wilson, D. M., 1965. Proprioceptive leg reflexes in cockroaches. *J. Exp. Biol.,* **43**: 397–409.

Wilson, D. M., 1966. Insect walking. *Ann. Rev. Entomol.,* **11**: 103–123.

Woodruff, M. L., P. Bownds, S. H. Green, J. L. Morrisey, and A. Shedlovsky, 1977. cGMP and the in vitro physiology of frog photoreceptor membranes. *J. Gen. Physiol.,* **69**: 667–679.

Woodruff, M. L., and S. M. D. Bownds, 1979. Amplitude, kinetics and reversibility of a light-induced decrease in guanosine 3′,5′-cyclic monophosphate in frog photoreceptor membranes. *J. Gen. Physiol.,* **73**: 629–653.

Woody, C. D., and P. Black-Cleworth, 1973. Differences in excitability of cortical neurons as a function of motor projection in conditioned cats. *J. Neurophysiol.,* **36**: 1104–1116.

Woody, C. D., D. Carpenter, J. D. Knispel, T. Crow, and P. Black-Cleworth, 1974. Prolonged increases in resistance of neurons in cat motor cortex following extracellular iontophoretic application of acetylcholine (ACh) and intracellular current injection. *Fed. Proc.,* **33**: 399.

Woody, C. D., and J. Engel, Jr., 1972. Changes in unit activity and thresholds to electrical microstimulation at coronal-pericruciate cortex of cat with classical conditioning of different facial movements. *J. Neurophysiol.,* **35**: 230–241.

Woody, C. D., J. D. Knispel, T. J. Crow, and P. A. Black-Cleworth, 1976. Activity and excitability to electrical current of cortical auditory receptive neurons of awake cats as affected by stimulus association. *J. Neurophysiol.,* **39**: 1045–1061.

Woody, C. D., and P. J. Yaronsky, 1972. Conditioned eye blink using electrical stimulation of coronal-precruciate cortex as conditional stimulus. *J. Neurophysiol.,* **35**: 242–252.

Woollacott, M. H., and G. Hoyle, 1977. Neural events underlying learning in insects: changes in pacemaker. *Proc. R. Soc. Lond. B. Biol.,* **195**: 395–415.

Zucker, R. S., 1972a. Crayfish escape behavior and central synapses. I. Neural circuit exciting lateral giant fiber. *J. Neurophysiol.,* **35**: 599–620.

Zucker, R. S., 1972b. Crayfish escape behavior and central synapses. II. Physiological mechanisms underlying behavioral habituation. *J. Neurophysiol.,* **35**: 621–637.

Zucker, R. S., D. Kennedy, and A. I. Selverston, 1971. Neuronal circuit mediating escape responses in crayfish. *Science,* **173**: 645–650.

Reference: *Biol. Bull.*, **159**: 561–570. (December, 1980)

THE FUNCTIONAL SIGNIFICANCE OF THE LUNULES IN THE SAND DOLLAR, *MELLITA QUINQUIESPERFORATA*

DAVID E. ALEXANDER AND JOE GHIOLD

Department of Zoology, Duke University, Durham, NC 27706;

and

Institut und Museum für Geologie und Paläontologie,
Universität Tübingen, Sigwartstrasse 10, 7400 Tübingen 1, West Germany

ABSTRACT

Experiments indicate that the lunules in *Mellita quinquiesperforata* function as food gathering devices. Allometric analysis of dead specimens shows that the lunules grow faster than does the body as a whole. This is analogous with the situation in certain fossil brachiopods, in which the lophophore (feeding structure) grows faster than the body. Field experiments involving sand dollars with plugged lunules show that righting and burrowing are not affected. However, animals with plugged lunules cannot feed as well as normal individuals, based on analysis of stomach contents. Thus, the lunules play an important role in feeding, although the exact mechanism has not been conclusively demonstrated. Our study, as well as previous work, suggest that the lunules act as a site for the passage of food particles from the aboral (upper) surface to the oral (lower) surface. Flow tank observations suggest that the lunules may act as devices to bring water, and thus food particles, up from the sand below the animal. In scutellid sand dollars, the fossil record shows an increase in lunule number over time. In Western Atlantic species, there is also an increase in lunule number with decreasing latitude. Perhaps these correlations reflect progressive adaptation by sand dollars to less productive waters.

INTRODUCTION

The slots and notches termed "lunules" are structures common to many scutellid sand dollars. Many authors (Berrill and Berrill, 1957; Hyman, 1958; Bell and Frey, 1969; Barnes, 1974) have speculated upon the function of the lunules, but there has been little experimental work. Ikeda (1941) found that specimens of *Astriclypeus* if their lunules are plugged cannot turn upright when inverted, and burrowing speed is impaired. However, Hyman (1958) and Ghiold (1979) observed that *Mellita quinquiesperforata* does not use its lunules in burrowing, and Weihe and Gray (1968) found that *M. quinquiesperforata* does not use its lunules in righting when inverted. The suggestion of Berrill and Berrill (1957) that the lunules strengthen the connection between the aboral and oral skeletal surfaces seems unlikely, as the internal calcareous columns already provide such support (Hyman, 1958). Furthermore, some scutellid sand dollars lack lunules entirely. Many workers have suggested other functions for the lunules in *M. quinquiesperforata.* These suggestions include removal of feces via upward

Received June 8, 1979; accepted September 25, 1980.

movement of sand through the lunule (Bell and Frey, 1969); maintenance of "direct communication with the substrate surface" by buried sand dollars (Bell and Frey, 1969, p. 557); and maintenance of a slightly inclined posture by passing sand downward through the anal lunule (Barnes, 1974). Podia and miliary spines, noted to function in food gathering, are concentrated along the inner walls of the lunules of *M. quinquiesperforata.* This characteristic led Ghiold (1979) to conclude that the lunules in this species are involved with feeding.

We studied the function of lunules in *M. quinquiesperforata* by analysis of lunule growth and development, and by observation and experimentation on living and dead specimens.

MATERIALS AND METHODS

Lunule growth analysis

A growth analysis was conducted to determine possible allometric relationships for the morphology of the lateral, posterior, and anal lunules. Seventy-four dead tests were collected for study along the intertidal margin of Bird Shoal, a small tidal flat in the Beaufort Inlet area of North Carolina. The maximum diameter of the echinoid test, measured along the anterior-posterior axis, was used as the prime index for sand dollar size. The maximum diameters for the sample population ranged from 98 to 32 mm. Lunule length measurements were plotted against this index to isolate those features displaying allometric growth. (This relationship does not account for test diameters smaller than 32 mm and is valid only for those specimens measured.) Measurements were recorded in millimeters with the aid of vernier calipers.

A computer program was designed to handle calculations from which a standard logarithmic plot was generated using the power function, $y = bx^a$. The program plotted the log of lunule size against the log of test size and drew a least-squares regression line through the data points for each graph. The joint mean, "D" (67 mm test diameter) was selected as an objective if somewhat arbitrary estimation of the beginning of the adult population. A dashed line which has a slope, "a" in the equation above, of 1.0 was drawn through "D" and represents a line of isometry (Gould, 1977). The resulting graphs are shown in Figures 1 and 2.

The slopes of all regression lines were tested to see whether lunule size changes disproportionately with changing body size. A multivariate regression technique was used to test this; Bonferroni simultaneous confidence intervals (Morrison, 1976) were calculated for the slope of each line with log (test diameter) as the independent variable and log (lunule length) as the dependent variable. This statistical treatment is not unbiased, because least-squares regression assumes that there is no error in the independent variable. Both our variables probably have about the same error, but any error in the x-direction artificially lowers the regression coefficient, making the test of whether the slope is different from 1.0 conservative if the calculated slope is greater than 1.0.

Flow tank experiment

For this experiment, dead tests of *M. quinquiesperforata* were collected by hand near Bird Shoal and preserved specimens were obtained from Carolina Biological Supply, Inc. Specimens were tested in a small Plexiglas flow tank as described

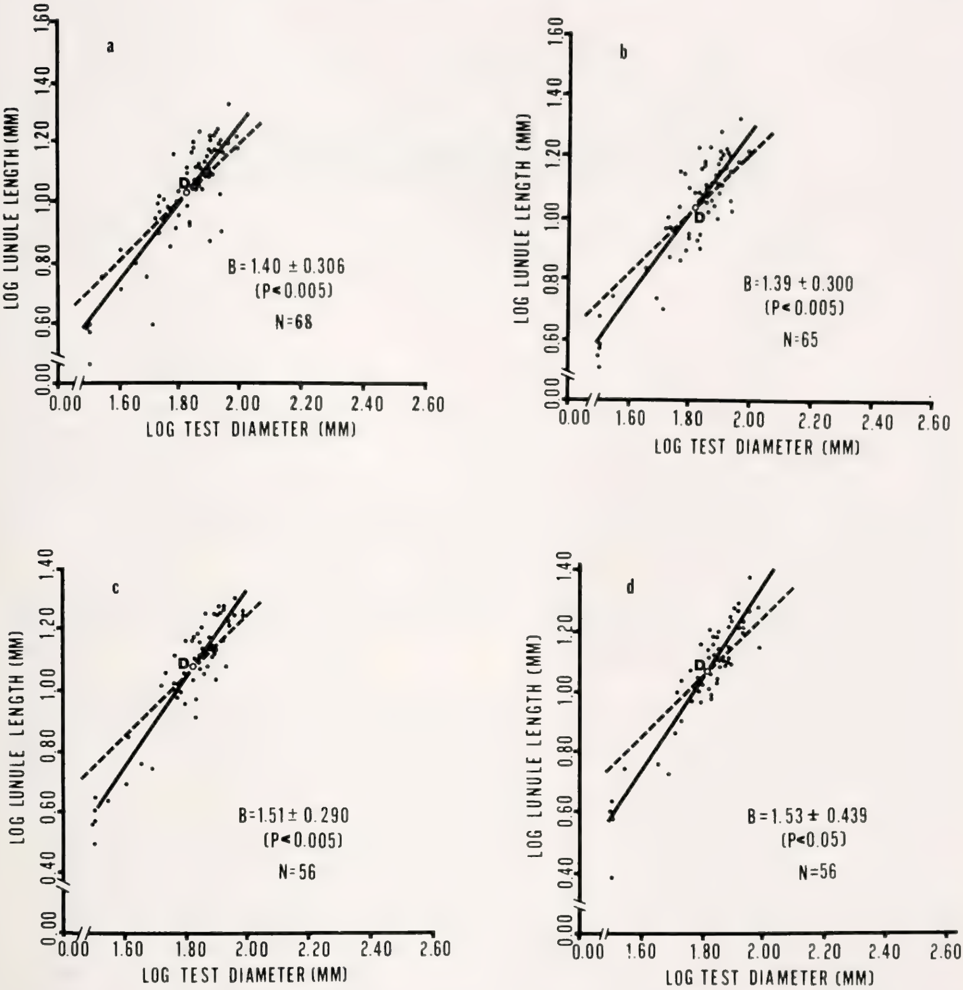


FIGURE 1. Length of left (a) and right (b) lateral lunules, and left (c) and right (d) posterior lunules measured against test length. Solid line is least-squares linear regression line; dashed line is a line of isometry (slope = 1.0) through the point D, estimating the beginning of the adult population (log (67 mm) or 1.83 on the x-axis). B: slope of regression line ± Bonferroni confidence interval at the indicated level of significance. N: sample size, which includes some specimens for which one or more lunule could not be measured; only 43 specimens with all lunules intact were used to calculate the multivariate confidence intervals.

by Vogel and LaBarbera (1978). Sand dollars were placed on a bed of sand and covered with a thin layer of sand. After injecting dye (Rotamine B) under the test, observations of flow patterns were made at current speeds ranging from 5–15 cm/sec.

Field experiment

In situ experiments were carried out on sand dollars along Middle Marsh, near Beaufort, North Carolina. Sand dollars were collected from an abundant population on a sand bar near Middle Marsh. Only large, healthy-looking sand dollars

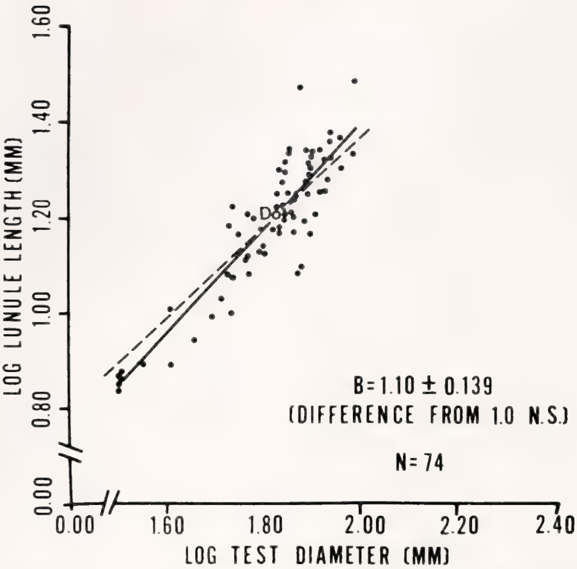


FIGURE 2. Length of the anal lunule measured against test length. Solid line is least-squares regression line, dashed line is line of isometry (see Fig. 1 for further explanation). Note slope confidence limits include 1.0.

were used. In long-term experiments individuals were marked by tying colored threads through the lunules and were positioned near the anchor of a float. By carefully raking the sand around the float anchor where the animals were placed, over 90% of the organisms were recaptured routinely.

The burrowing speed of each of five sand dollars was measured two times before and two times after the lunules were plugged. We found that the time it takes an uncovered sand dollar to become completely covered with sand is an excellent indicator of burrowing speed; thus, the burrowing time of several sand dollars was measured before and after plugging the lunules with soft paraffin.

To determine whether the lunules are used in righting inverted sand dollars, 12 sand dollars were collected and half of them had their lunules plugged. The sand dollars were left inverted near the float anchor and observed at the next low tide (for unknown reasons, *Mellita quinquiesperforata* will right itself in the field but not in the laboratory.)

To test the importance of lunules in feeding, 24 sand dollars were collected from the area near the float anchor and tagged. Twelve had their lunules plugged with soft paraffin, and the remaining animals were used as controls. Each control individual was exposed to air for the length of time that it took to plug the lunules of an individual in the other group. All the animals were returned to the sand near the float anchor within a few meters of where they were collected. Two days later, the sand dollars were recaptured. Animals were killed in the field by immersion in fresh water so as to minimize movement of the stomach contents. The organisms were then returned to the laboratory and placed in 95% ethanol for 4–6 days to harden their tissues. Stomachs were dissected out, placed on glass slides, and dried overnight at 100°C. The stomachs were then weighed to the nearest 0.0001 g on a Mettler balance. The laboratory work in this experiment was carried out at Duke University Marine Laboratory, which also provided field equipment.

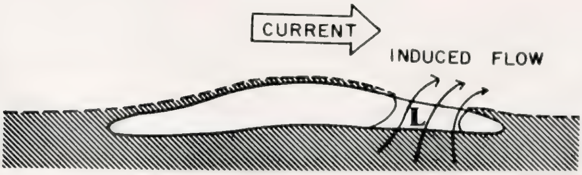


FIGURE 3. Illustration of flow tank experiment results. Schematic cross-section through the sand dollar shows water moving up through the lunule in response to a current.

RESULTS

Lunule growth analysis

The population measured for lunule growth exhibited an allometric relation for the length of the two lateral and two posterior lunules (Fig. 1). The anal lunule showed a non-significant allometric trend in the same direction (Fig. 2). The slopes of the regression lines are shown with the Bonferroni confidence intervals on the figures. The allometric relationships suggest that the lunules grow more rapidly than does the body. We feel confident that this is a real developmental pattern and not an artifact of adult size distribution, because sand dollar growth appears to be highly indeterminate. There is at least a two- or three-fold range in adult size (personal observation) and there is no reason to believe that large adults are the same age as small adults.

There may be no functional significance in the allometric trend alone, but the allometry raises the possibility that the functional importance of the lunules differs as body size changes.

Flow tank experiment

The flow tank study illustrates an unusual property of the lunule: When an appreciable current (8 cm/sec or more) was passed over the sand dollar, water from the sand beneath the lunule was drawn up through the lunule (Fig. 3). This was probably due to a combination of Bernoulli effect and viscous entrainment, what Vogel (1977) has called "induced flow." Although the process occurred to a small extent at very low current speeds, at higher speeds—above about 8 cm/sec—a significant stream of dye appeared in a few seconds.

Field experiments

We found strikingly little difference in the burrowing speed of five sand dollars before and after plugging their lunules: The mean time for animals with open lunules was 230 sec (s.e. ± 10.6); for the animals with plugged lunules, it was 231 sec (s.e. ± 18.0). Also, the righting ability of sand dollars with plugged lunules did not seem to suffer: of the six sand dollars with plugged lunules and six with open lunules that were inverted in the field, all 12 were right-side-up by the next low tide.

Data from the feeding experiment show that for a given body size, sand dollars with plugged lunules had lighter stomachs than sand dollars with normal lunules (Fig. 4). Sand dollars have flat, translucent stomachs, and visual inspection explained the weight difference: 11 of the 12 control animals had stomachs approximately 1/4 to 3/4 full. In contrast, eight animals with plugged lunules had completely empty stomachs, and the two others recaptured had far less in their stomachs than any of the controls.

Unexpectedly, paraffin from one lunule of a plugged specimen was missing upon recapture. When the stomach contents of this specimen were weighed, they clearly fell in the range of sand dollars with open lunules (Fig. 4). This strongly suggests that the paraffin itself has no damaging effects on sand dollar feeding behavior, other than to stopper the lunules.

Discussion

Our burrowing data show that *Mellita quinquiesperforata* does not use its lunules in burrowing. Unlike Ikeda's (1941) observations, our righting experiment clearly shows that open lunules are not necessary for righting. Unfortunately, we could not watch the sand dollars over a whole tidal cycle in the field, so we do not know if plugging the lunules slows righting.

Our evidence, although indirect, strongly suggests that the lunules of *M. quinquiesperforata* are involved in the food gathering process and apparently expedite the transfer of food particles to the mouth.

Our observations on the lunules are at variance with the observations of Bell and Frey (1969) as well as Ikeda. Bell and Frey suggested that the lunules somehow and for some unknown reason function to keep the echinoid in contact with the surface of the sand, based on small pits in the sand over the lunules. We observed these pits commonly over animals beached by the falling tide, but very rarely in normal burrowing animals. Also, Bell and Frey's description of sand dollars pushing sand up through the large anal lunule and leaving a well-defined cylinder of sand behind the organism is unlike any behavior Hyman (1958), Weihe and Gray (1968), or we observed. From observations of specimens in the field and in the laboratory, we noticed that sand dollars occasionally pump with the anal papilla just enough to clear a small portion of the anal lunule of sand and eject a cloud of flocculent fecal matter; at no time did we observe a significant amount of sand passing up through any of the lunules. In fact, as the sand dollars burrow, they characteristically raise the spines around the lunules into a fence-like structure so that sand must pile up until it is high enough for some grains to fall over into the lunule.

Field experiments in our study strongly suggest that the lunules are involved in feeding. There are several ways the lunules may function as feeding devices. They

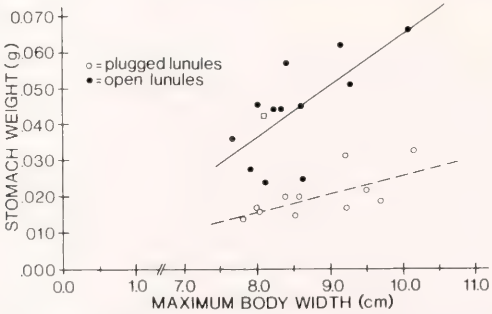


Figure 4. Results of the feeding experiment show that for a given body size, animals with plugged lunules (open circles) have lighter stomachs than control animals (closed circles). A sand dollar with only four of its five lunules plugged is indicated by the open square. Lines were obtained by linear regression.

may be sorting and collecting areas for food particles trapped on the aboral (upper) surface (Ghiold, 1979) and may function as an additional "edge" to pass particles from the aboral to the oral side as observed in *M. sexiesperforata* by Goodbody (1960). Another possibility is suggested by our flow tank study: The sand dollars may be using induced flow (Vogel, 1977) to draw water and small particles up from the underlying sand. Presumably, the water then passes through the lunule, where spines and podia trap potential food particles. As it is known that water flows down into and up out of ripples in a sand or gravel bed under a current (Webb and Theodor, 1972), even a sand dollar buried too deeply to take advantage of induced flow could passively exploit the movement of water through the substrate in the manner described for induced flow.

The allometric growth of lunules also suggests that lunules are involved in feeding. Our data show that lunules grow faster in length than does the animal's body. Ghiold (1979) has demonstrated that the spines of the body and lunule margins are well suited for food collecting. Seilacher (1979) mentioned that lunules may result in an allometric increase in the spine-fringed margin or edge, and he commented that the margin must have an important food gathering role. Seilacher also noted that some sand dollars seem to show a negative allometry of the lunule width. If the lunule is approximated by a long, narrow rectangle, elementary geometry shows that with negative allometry of the width, positive allometry of the length can still produce an allometric increase in perimeter. As the perimeter of the lunule seems to be the lunule's important food gathering characteristic, any negative allometry of the width is probably unimportant relative to the positive allometry of the length. The allometric increase in perimeter is important because the body increases considerably in weight for a given increase in length, although sand dollars are so nearly two-dimensional that the square: cube relationship is not a good approximation. But even considering the extreme case of weight being proportional to area, the area still increases π times the increase in length. Thus, assuming the weight-specific metabolic rate stays constant, a sand dollar's appetite will always grow faster than its linear dimensions. A similar relationship is found in some fossil brachiopods: the lophophore, an internal food gathering structure, increases in relative size and complexity with increasing body size for *Aemula inusitata,* a species from the Cretaceous of Northwestern Europe (Gould, 1977). Thecidelliniform brachiopods from the Jurassic display similar feature changes (Gould, 1966).

The geographic distribution of recent scutellid sand dollars along the North American east coast suggests a possible relationship between climate and lunule number. *Echinorachnius parma* is a cold-temperate species found in New England waters and has no lunules. *M. quinquiesperforata,* a mild-temperate to tropical species found south of Cape Hatteras, North Carolina, has five lunules (very rarely, six). *M. sexiesperforata,* which has six lunules, and *Encope michlini,* which has five notches and one very large anal lunule, are found in tropical regions and are extremely rare in temperate areas (Kier and Grant, 1965). One environmental factor that varies between cold and tropical waters is the nutrient level: cold waters tend to be richer in nutrients than tropical waters (Russell-Hunter, 1970). Perhaps the presence of lunules is a response to the reduced food supply.

Lunules and notches (incomplete lunules) may represent an evolutionary modification to enhance food gathering in sand dollars. As Seilacher (1979) pointed out, the change in plate growth pattern from the regular urchins to that

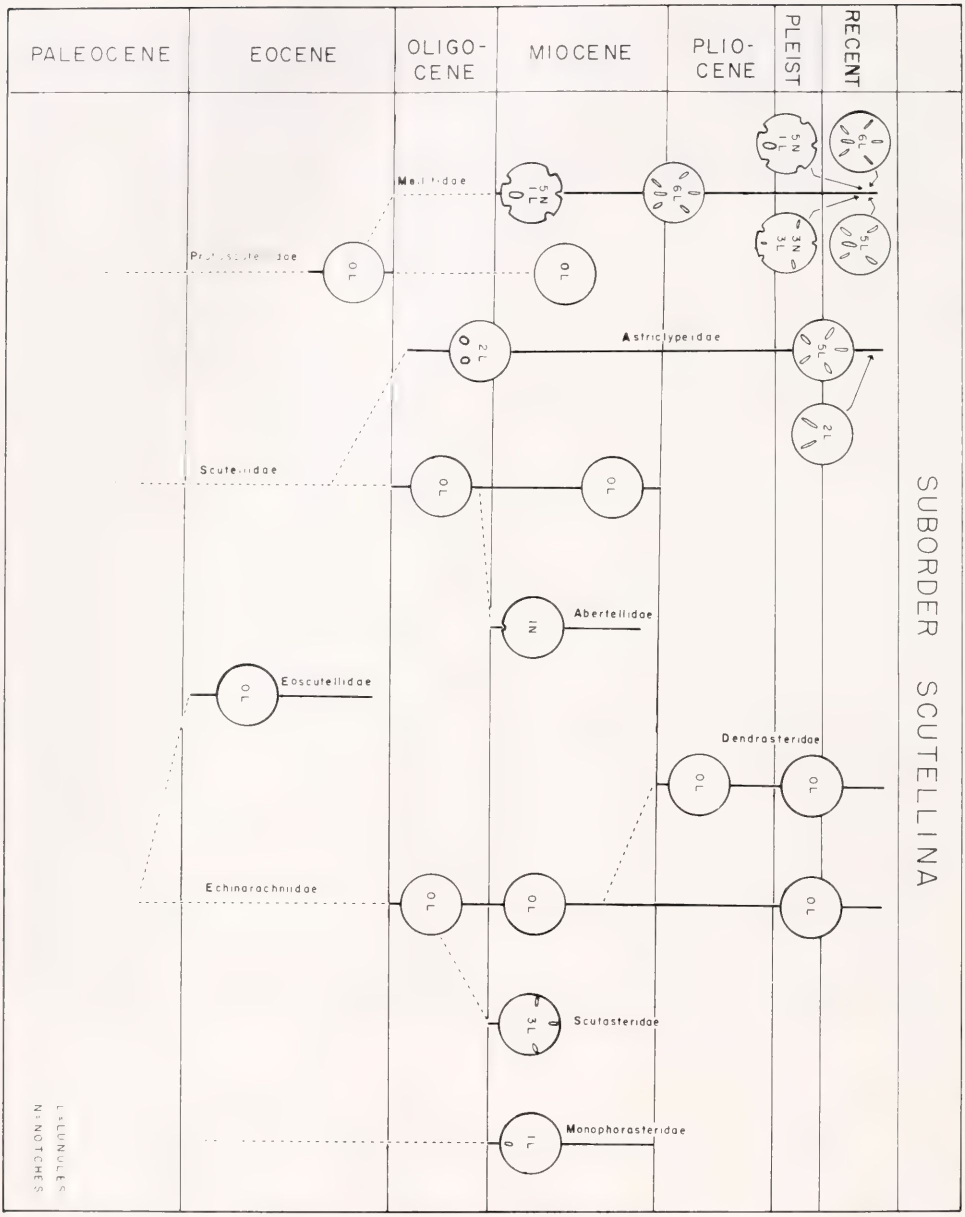

PALEOCENE
EOCENE
OLIGO-CENE
MIOCENE
PLIO-CENE
PLEIST
RECENT
Mellitidae
5N 1L
6L
5N 1L
6L
3N 3L
5L
Protoscutellidae
0L
0L
2L
Astriclypeidae
5L
2L
Scutellidae
0L
0L
1N
Abertellidae
0L
Eoscutellidae
Dendrasteridae
0L
0L
Echinarachniidae
0L
0L
0L
3L
Scutasteridae
1L
Monophorasteridae
SUBORDER SCUTELLINA
L=LUNULES
N=NOTCHES

FIGURE 5. Evolution of lunules in scutellid sand dollars. Vertical spacing proportional to time except for Pleistocene. This diagram is modified from Durham (1966, p. 453) notably in the family Mellitidae where *Mellita aclinensis, M. Sexiesperforata, M. quinquiesperforata* and the genus *Encope* are depicted. The genus *Echinodiscus* is also shown in the family Astriclypeidae.

of the irregular urchins allowed new constructional possibilities such as lunules. The important role of the lunules is emphasized by the fact that lunules appear to have arisen independently in six different sand dollar lineages (Seilacher, 1979). According to the fossil record the first lunules appeared during the Oligocene in the suborder Scutellina, and over time, lunules increased in number and development, especially within the family Mellitidae (Fig. 5). One exception to this trend is the descent of the modern *Mellita quinquiesperforata* from *M. aclinensis*. *M. aclinensis* was a six-lunuled fossil species, which lived during the late Miocene and Pliocene in warm, subtropical waters (Kier, 1972). Kier believes that the mild-temperate *M. quinquiesperforata* evolved directly from the six-lunuled tropical species *M. aclinensis* to its modern form with commonly five, but rarely six, lunules. Thus, the fossil record also shows a correlation of lunules with warm climates, and perhaps with food supply.

Lunules and notches of *M. sexiesperforata* and *Encope michelini* were not observed, but the distribution of these external perforations and indentations is so similar to that of *M. quinquiesperforata* that they undoubtedly function in the same manner. We emphasize, however, that further examination of other sand dollars is necessary before the ideas presented here can be applied to lunules in other families.

Acknowledgments

D. E. A. would like to thank Alexander Motten, Helen Miller, and Garth Ware for much helpful discussion, Diane Campbell for important statistical help, and Steven Vogel for his plentiful encouragement and support.

The section on lunule growth draws heavily upon parts of a senior thesis completed by J. G. while at Harpur College, SUNY-Binghamton. Instrumental in completion of that work and a source of stimulating discussion on growth analysis were Don L. Kissling and James R. Beebower. Steven R. Dickman provided valuable assistance with the computer program.

Both authors thank Robert D. Barnes, Gettysburg College; David L. Pawson, U. S. National Museum, and Patricia Timko Withers, Duke University, for critically reviewing the manuscript, and Adolf Seilacher, University of Tübingen, for commenting on revisions. Steven J. Gould, Harvard University, kindly identified himself as a reviewer and offered many pertinent suggestions for preparation of the final manuscript.

LITERATURE CITED

Barnes, R. D., 1974. *Invertebrate zoology,* 3rd edtn. W. B. Saunders Company, Philadelphia. 870 pp.

Bell, B. M., and R. W. Frey, 1969. Observations on ecology and the feeding and burrowing mechanisms of *Mellita quinquiesperforata* (Leske). *J. Paleontol.* **43**: 553–560.

Berrill, N. J., and J. Berrill, 1957. *1001 Questions Answered About the Seashore.* Dodd, Mead and Co., New York. 305 pp.

Durham, J. W., 1966. Clypeasteroids. Pp. 450–491 in R. C. Moore, Ed., *Treatise on Invertebrate Paleontology,* Part U, Echinodermata 3. Geol. Soc. Am. and Univ. Kans. Press, Lawrence, Kansas.

Ghiold, J., 1979. Spine morphology and its significance in feeding and burrowing in the sand dollar *Mellita quinquiesperforata* (Echinodermata: Echinoidea). *Bull. Mar. Sci.,* **29**: 481–490.

Goodbody, I., 1960. The feeding mechanism in the sand dollar *Mellita sexiesperforata* (Leske). *Biol. Bull.,* **119**: 80–86.

Gould, S. J., 1966. Allometry and size in ontogeny and phylogeny. *Biol. Rev.,* **41**: 587–640.

Gould, S. J., 1977. *Ontogeny and Phylogeny.* Belknap Press of Harvard Univ. Press, Cambridge, Mass. 501 pp.

Hyman, L. H., 1958. Notes on the biology of the five-lunuled sand dollar. *Biol. Bull.,* **114**: 54–56.

Ikeda, H., 1941. Function of the lunules of *Astriclypeus* as observed in the righting movement (Echinoidea). *Annot. Zool. Jpn.*, **20**: 79–82.

Kier, P. M., 1972. Upper Miocene echinoids from the Yorktown formation of Virginia and their environmental significance. *Smithson. Contrib. Paleobiol.*, **13**: 1–41.

Kier, P. M., and R. E. Grant, 1965. Echinoid distribution and habits, Key Largo Coral Reef Preserve, Florida. *Smithsonian Misc. Coll.*, **149(6)**: 1–68.

Morrison, D. F., 1976. *Multivariate Statistical Methods.* McGraw-Hill Book Co., New York. 420 pp.

Russell-Hunter, W. D., 1970. *Aquatic Productivity.* Macmillan Publishing Co., Inc. New York. 306 pp.

Seilacher, A., 1979. Constructional morphology of sand dollars. *Paleobiology,* **5(3)**: 191–221.

Vogel, S., 1977. Flows in organisms induced by movements of the external medium. Pp. 285–297 in T. J. Pedley, Ed., *Scale Effects in Animal Locomotion.* Academic Press, Inc., New York.

Vogel, S., and M. LaBarbera 1978. Simple flow tanks for research and teaching. *BioScience,* **28**: 639–643.

Webb, J. E., and J. Theodor, 1972. Wave induced circulation in submerged sands. *J. Mar. Biol. Assn. U. K.*, **52**: 903–914.

Weihe, S. C., and I. E. Gray, 1968. Observations on the biology of the sand dollar *Mellita quinquiesperforata* (Leske). *J. Elisha Mitchell Sci. Soc.*, **84**: 315–327.

Reference: *Biol. Bull.*, **159**: 571–581. (December, 1980)

THE URN CELL COMPLEX OF *SIPUNCULUS NUDUS*: A MODEL FOR STUDY OF MUCUS-STIMULATING SUBSTANCES[1]

BETSY G. BANG AND FREDERICK B. BANG

Department of Pathobiology, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland 21205, and Station Biologique, 29211 Roscoff, France

ABSTRACT

The secretory systems of the living urn cell complex of *Sipunculus nudus* were studied by light and fluorescence microscopy, using vital stains. The complex comprises two morphologically distinct secretory systems which respond to different stimuli, secrete at different rates, and respond differently to vital dyes. One system apparently resides in the ciliated base cell of the complex, the other in a cluster of small secretory "R" cells (presumptive regulatory cells) attached to the central membrane of the base cell. R cells slowly secrete mucus that is selectively sticky for cell debris and foreign particulates. When stimulated by pathogenic bacteria or other defined substances, the other system rapidly synthesizes streams of mucus from four to five synthetic loci. The loci are at rest until stimulated. *In vivo* the two secretory systems keep *S. nudus* coelomic fluid sterile and free of debris. Both systems respond *in vitro*. In *in vitro* suspensions, R cells are depleted over time; concomitantly, streams of mucus from stimulated loci become longer, suggesting a degree of regulation by R cells. R cells contain large granules of neutral red-staining material, but do not stain with Janus green B. The synthetic loci stain intensely with the purple form of Janus green B but do not stain with neutral red.

INTRODUCTION

The mucus-producing urn cell complexes of the marine invertebrates *Sipunculus nudus* respond *in vitro* to mucus-stimulating substances in the body fluids of humans and rabbits. There is a quantitative change in responses to the same fluids during the course of clinical disease (Bang and Bang, 1979). Light-microscopic studies of vitally stained living urn-cell complexes indicate that each of two secretory systems of the complex produces one or more morphologically distinctive types of mucus, each of which has a particular function. The present report reviews the functional dynamics of each secretory system as seen in vitally stained living urn-cell complexes. It also considers some of the variables inherent in the use of urn cell complexes as a biomedical assay system.

MATERIALS AND METHODS

Methods of collection and maintenance of *S. nudus*, of obtaining suspensions of urn cell complexes, and of observing the behavior of urn cell complexes *in vitro*

Abbreviations: BFSW—boiled filtered seawater; JGB—Janus green B; R cells—presumptive regulatory cells; DASPMI—diaminostyrylpyridinium-iodine.

Received June 26, 1980; accepted September 25, 1980.

[1] Supported by NIH grant #5 P50 HL-19157.

have been described (Bang and Bang, 1962). In the original studies (Bang and Bang, 1976), a series of vital dyes was used for the purpose of evaluating the specificity of a given dye for a given organelle. On the basis of those results, neutral red and Janus green were used to further define the separate characteristics of two apparently distinct secretory systems within the complex. For the present studies, a 1:500 solution of neutral red (Matheson, Coleman, and Bell) was prepared in distilled water, then diluted in boiled filtered seawater (BFSW) for a final dilution of 1:5000. Janus green B (JGB) (British Drug House) was prepared in the same way for a final 1:1000 solution, which was then twice filtered through a Whatman #1 filter.

The seawater used to dilute for dyes and sera in all experiments was natural seawater boiled for 5 min, twice filtered through #1 Whatman filters, and stored at 4°C.

The stock stimulus for eliciting secretion of the free-flowing mucus from the loci was human serum from the same donor, diluted 1:10 in BFSW and heated to 85°C for 6 min to produce a strong stimulus. Serial dilutions of this stock provided moderate to mild stimuli as required.

For fluorescence microscopy, a seawater buffered 0.01% solution of acridine orange was used to identify cell nuclei. For vital staining of mitochondria, a 2 μM solution of the specific vital mitochondrial probe diaminostyrylpyridinium-iodine (DASPMI) (Bereiter-Hahn, 1976) was prepared in BFSW. On a glass slide, 5 μl of fluorescent stain were added to 5 μl of supernatant urn cell complexes at room temperature, and the appearance of fluorescence was monitored at 5-min intervals. Both acridine orange and DASPMI showed some fluorescence at 20 min, and reached sustained brightness by 40 min. To stimulate cells, 5 μl of a 1:2 dilution of stock stimulus was added simultaneously with the dye. All fluorescent preparations were stained and photographed at the Station Biologique by Dr. Christian Sardet, Groupe de Biologie Marine, Station Zoologique, Villefranche-sur-Mer.

Results

The urn cell complex.

Free urn cell complexes are normal components of the coelomic fluid (blood) of *S. nudus.* They originate as fixed epithelial cell complexes in the coelomic-lining epithelium, where they function to trap and remove particulate debris and to hypersecrete mucus in response to bacterial pathogens. Thousands of fixed urn cell complexes periodically detach from the body wall and become free-swimming in the blood, where they perform the same functions.

By producing two types of mucus, the secretory cells of the complex participate in three known major functions in the living animal: removal of foreign and cellular debris, cell clotting, and secretion of mucus that immobilizes and isolates bacteria. Non-stimulated, healthy, autologous cells are never entrapped in urn-cell-complex mucus. All of these responses are reproducible *in vitro* in suspensions of urn cell complexes maintained in their own coelomic fluid.

The complete urn cell complex (Fig. 1) ranges in diameter from about 65–75 μm. It was first understood to consist of two cells, a "vesicle cell" and a mucociliated base cell (Bang and Bang, 1965). But preliminary electron microscope observations by Reissig (Reissig *et al.*, 1979) have shown that the complex comprises three cell types: a "vesicle cell," a mucociliated base cell, and a cluster of small

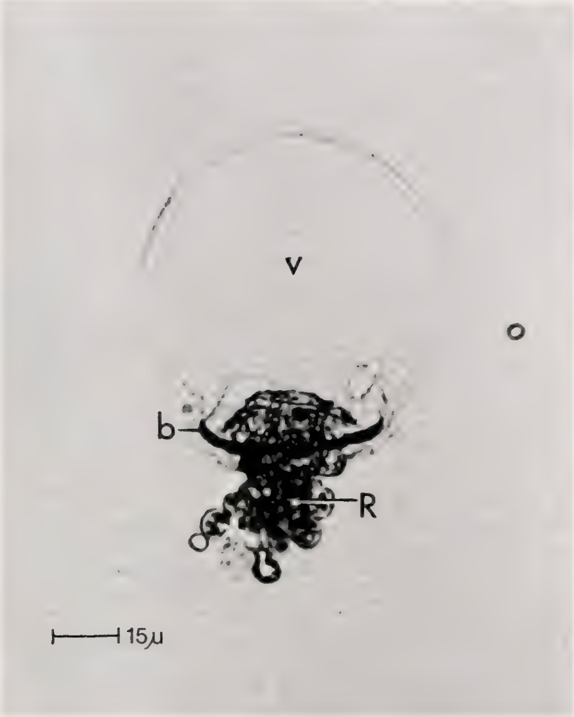


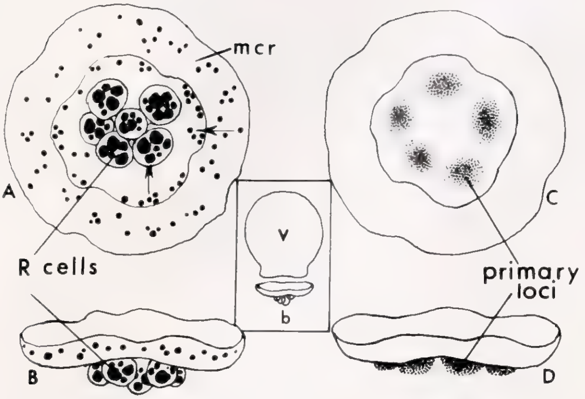
FIGURE 1. Profile of unstained urn cell complex: vesicle cell (v), base cell (b), and R cells (R) covered by adherent amebocytes. Cilia do not show.

(about 2 μm) secretory cells attached to the external membrane of the base cell (Fig. 2). The vesicle cell is very thinly stretched to form a transparent hollow dome (Fig. 3, inset), the base of which is attached to the upper rim of a quadrifoil saucer-shaped mucociliated base cell by junctional complexes. The two cells thus enclose a transparent matrix. Attached to the center of the outer membrane of the base cell, also by junctional complexes, is the cluster of small secretory R cells (so designated in honor of Reissig). Between the R cells and the mucociliated rim of the base cell are several (usually four) loci of synthesis of free-flowing mucus secreted in response to defined stimuli. The static and active relationships are diagramed in Figures 2 and 3.

There is an apparent division of labor between the two secretory systems: the R cells continually and slowly produce brief tails of granular mucus that is selectively sticky for cell debris and foreign particulates; the other system is at rest until the loci on the nonciliated portion of the base cell membrane are stimulated to secrete streamers of free-flowing mucus.

Evidence for two separate secretory systems

The R cells. If a drop of fresh whole blood from *S. nudus* is diluted about 1:20 in seawater and put on a glass slide, it can be seen that the rapidly swimming urn-cell complexes have few, if any, autologous cells adherent to them. However, in fresh preparations of supernatant urn cell complexes, the R cells nearly always have moderate loads of adherent amebocytes (Fig. 1), presumably activated as a



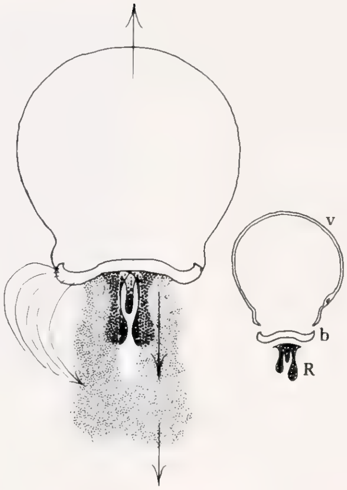


FIGURE 2. (Left) Diagram of base cell of urn cell complex, viewed from exterior surface and in profile: A. R cells in center of non-ciliated membrane; muco-ciliated rim (mcr), cilia not drawn; B, profile, secretory granules indicated by black dots; C, position of hypersecretory loci in central membrane; D, profile. Inset: relationships of vesicle (v) and base cell (b) with attached R cells.

FIGURE 3. (Right) Diagram of interrelations of R cells and actively secreting loci, urn cell complex swimming forward (arrow on vesicle cell) while secreting tail emerges (arrows) pulling R cells with it for varying distances. Inset: relationship of vesicle cell (v), base cell (b), and R cells, shown as though junctional complexes were removed.

result of bleeding. When these adherent cells are removed by centrifugation at 12,000 rpm for 1 min, the cluster of R cells and tags of the secretions are revealed (Fig. 4). From 80 to 100% of active urn cell complexes from freshly bled healthy *S. nudus* have clusters of 4–7 adherent R cells.

Neutral red staining of urn cell complexes in individual suspensions over time has shown that beginning late in the first week and continuing for the subsequent 3–4 weeks, R cells gradually detach from the base cells of individual urn cell complexes (Table I). During this gradual *in vitro* loss of R cells, the tails of mucus induced in the free-flowing locus system change visibly, generally becoming longer and slimmer with time. In about 5% of suspensions, the R cells persist, and foreshortened tails predominate for the life of the suspension. Thus the presence of the full complement of R cells evidently has some control over the quality of free-flowing secretions *in vitro*. Whether R cells may be replaced *in vivo* is not known.

The large packages of granules in R cells stain brilliantly with neutral red, so that it is easy to follow the behavior of R cells as their membranes stretch when, during response to a strong stimulus, they are pulled distalward by traction of the emerging locus secretions. The nuclei are in the distal portion. The membrane often stretches until it breaks, and the nuclear portion is cleaved from the base cell. We had originally interpreted the R cells as independent sacs of the base cell membrane, possibly mucus reservoirs (Bang and Bang, 1965), but Dr. Reissig's electron-microscopic studies revealed that they are separate cells (Reissig *et al.*, 1979). The fact that R cell nuclei remain in the distally stretched portion of R cells during hypersecretion of the free-flowing system was confirmed by acridine orange vital staining (Fig. 5).

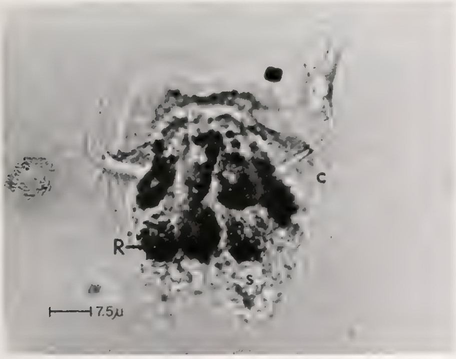
TABLE I

Loss of R cells from four preparations of supernatant urn cell complexes, three monitored over time. The numbers of visible R cells on 100–110 urn cell complexes were counted at random, using a cell counter. Ages of individual S. nudus *unknown.*

Preparation #	Days in culture	>3 R cells (%)	<2 R cells (%)	Lacking R cells (%)
1	1	80	20	0
	3	60	37	2
	5	56	35.5	8.5
	7	32	61	8
	10	14	71	15
2	11	36	54	10
3	13	14	49	38
	17	0	24	77
	19	0	2.5	97.5
4	21	2	54	44
	25	0	52	48
	28	0	46	53
	31	0	21	83

Cleavage of R cells from the base cell. Not only were R cells gradually detached from base cells over time in culture, but they were observed to be cleaved from the base cell *in vitro* by bacterial action. Further, Dr. Moon Shin, Associate Professor of Pathology, University of Maryland School of Medicine, was able to induce subtotal cleavage of R cells from the base cells of stimulated urn cell complexes by incubating them with neuraminidase prior to stimulation.

S. nudus spends much of its life in partially anaerobic conditions in burrows under the sand of the ocean floor. We investigated the capability of urn cell complexes to respond *in vitro*, over time, to a mild heated-serum stimulus after



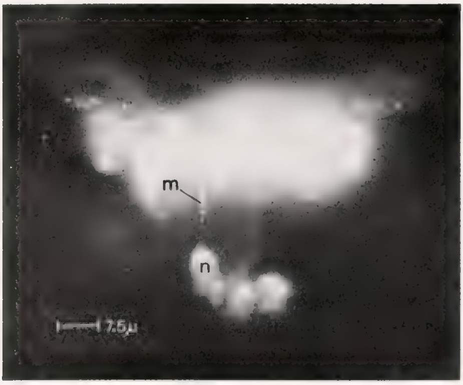


FIGURE 4. (Left) Lower portion of urn cell complex killed by formalin gas, showing immobilized cells (c), R cells with secretory granules stained with neutral red (R), and granular secretions revealed after removing attached amebocytes by centrifugation.

FIGURE 5. (Right) Stretched R cells of stimulated living urn cell complex, stained with acridine orange, showing R cell nuclei (n) and stretched membranes (m) with enclosed secretory granules. Fluorescence microphotograph by Dr. Christian Sardet.

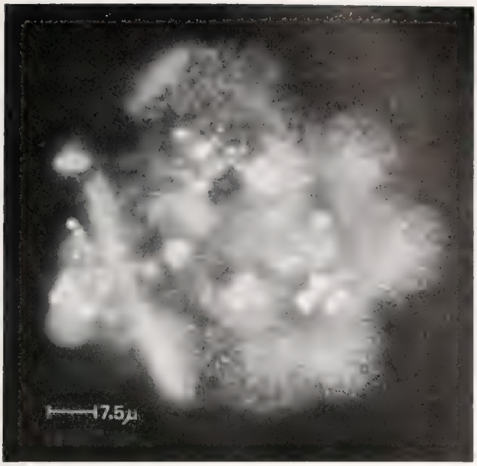
they were placed in a sealed flowing-nitrogen gas chamber. A small vial containing a 1-day-old suspension of urn cell complexes with full complements of R cells, and another vial containing the stimulus, were placed in the chamber for 2 hr; then at hourly intervals for the next 6 hr 10 μl of stimulus were added to 10 μl of the complex-containing suspension in a depression slide under flowing nitrogen. A coverglass was sealed in place by silicone, and responses were immediately observed in the light microscope. After 4 hr under nitrogen, many R cells appeared swollen, and R cells were seen to be pushed distally and detached from the base cell. By the 6th hour, rampant bacterial infection was present in both the suspension of urn cell complexes and the stimulus preparation; there were no R cells on the urn cell complexes, and the base cell loci were hypersecreting thin mucus at very rapid rates.

In Dr. Shin's unpublished experiments, newly bled urn cell complexes were incubated for 3 hr in *Vibrio cholerae* neuraminidase (2 units/ml in BFSW). Then a moderately strong human serum stimulus was added. Cells were killed after they had secreted for 10 min, and those which showed stretching of R cell membranes and cleavage of the nuclear portion of the cell were counted. Only 1% of control complexes showed a lack of R cells, while 64% of the enzyme-treated ones showed separation of the nuclear portion of R cells from the base cell by breakage of the over-stretched cell membrane. As in urn cell complexes, which lose R cells over time, the stimulus-induced tails subsequently produced by complexes from which R cells had been cleaved were two to three times longer than those of complexes which had retained the R cells. There was no stretching of R cells in unstimulated complexes.

R cell pathology. The only condition thus far identified as one in which R cell hypersecretion occurs is a transmissible pox-like skin disease of *S. nudus* which sometimes appears in laboratory-maintained animals. R cell abnormalities were found in coelomic fluid samples from eight *S. nudus* experimentally infected with the skin disease. In five cases there was R cell hypertrophy, in one case marked excess R cell secretion, and in the other two cases R cells were absent and possibly cleaved. During these events, there was no evidence of any activity from the hypersecretory loci. The serum of animals infected with this disease was consistently found to contain a previously identified heat-labile lysin against the ciliate *Anophrys* (Bang, 1966).

The hypersecretory loci. While R cell intracellular granules stain intensely with neutral red, neither the granules nor the extracellular mucus stains with Janus green. The loci which produce free-flowing mucus in response to specific stimuli do not stain with neutral red, nor does the extracellular mucus which emerges from these loci. The loci do, however, stain within a few minutes of exposure to the vital basic azo dye Janus green, concentrating the purple (reduced) form of the dye (Bang and Bang, 1976). The dye kills the urn cell complexes unless the loci are stimulated to produce mucus. The dye is evidently metabolized in some way during the secretory process and is re-excreted with the mucus, expressing the color which indicates the reduced or oxidized form of the dye (Lazarow and Cooperstein, 1953) depending on the stimulus. This dye is known to be preferentially adsorbed on the insoluble protein fraction of serum albumin (Lazarow and Cooperstein, 1953).

R cells and loci: separate secretory systems. Three sets of light microscope evidence indicate that the R cells and loci are separate secretory systems.



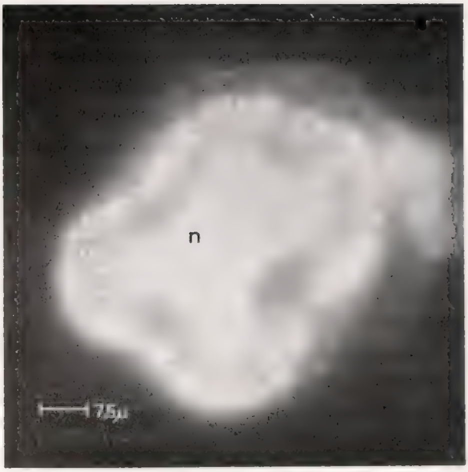


FIGURE 6. (Left) Mucociliated rim of base cell of 1-day-old living urn cell complex 30 min after staining with the mitochondrial fluorescent stain DASPMI, showing the lacework of mitochondria. Fluorescence microphotograph by Dr. Christian Sardet.

FIGURE 7. (Right) Same area of base cell of living 20-day-old urn cell complex, showing small rounded mitochondria. Pale oval is nucleus (n), blank areas are presumably loci. Fluorescence microphotography by Dr. Christian Sardet. DASPMI stain.

First, when new suspensions of urn cell complexes were incubated in neutral red in direct sunlight for 90 min, by which time the stained R cells were presumably partly inactivated, the loci immediately concentrated Janus green and hypersecreted vigorously in response to the stock stimulus.

Second, in 20–25 day suspensions in which many complexes visibly lacked R cells, the loci also quickly concentrated Janus green and subsequently secreted when stimulated.

Finally, when complexes that lacked R cells were stimulated with either serous nasal fluid or centrifuged lacrimal fluid, each of which induced a separate refractile stream of secretion, the point of attachment of each stream to its locus of origin on the base cell was clearly visible at 400 × magnification. The ultrastructure of both of the secretory systems awaits electron microscopic analysis.

Mitochondria. The base cell of the urn cell complex is very active metabolically, since the cilia and interciliary secretory granules are in the outer rim of this cell.

The specific fluorescent mitochondrial stain DASPMI revealed the extremely rich lacework of mitochondria in this area in freshly cultured urn cells (Fig. 6). The mitochondria in the central membrane of the cell shown were obscured by nonspecifically fluorescent R cells and adherent amebocytes.

Figure 7 shows the same area in a 20-day-old urn cell complex that lacked both R cells and adherent amebocytes: the separate, rounded mitochondria of the central membrane occupy all areas except those taken up by the large nucleus and by the presumptive loci, which appear as blank spaces.

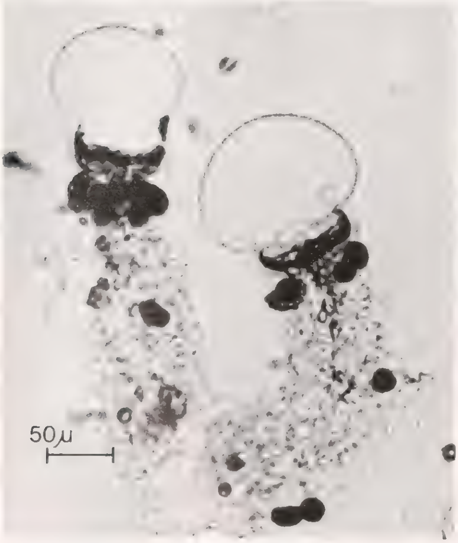
During observation of this moribund old cell, the vesicle became separated from the base cell and revealed a remarkable concentration of mitochondria in the rim of the vesicle at its site of attachment to the base cell.

Interactions of R cell and locus secretions. We have not yet been able to develop a stimulus which clearly induces concomitant R cell and locus secretions. When autologous heat-killed erythrocytes were added to a new suspension of urn cell complexes during active secretion from the loci, the red cells tended to adhere predominantly to the proximal part of the secreted tail at the level of the R cells (Fig. 8), suggesting that polymerization of some R cell product may interact with the freely flowing secretion at that level.

Effect of S-H compounds. Because of their capacity to break S-H bonds in respiratory, intestinal, and cervical mucus, the cysteine compounds are frequently used to solubilize mucus for therapeutic and experimental purposes. We are interested in the effects of these amino acid compounds on the synthesis of mucus by urn cell complexes and in their effects on previously secreted or final mucus.

Incubation of a suspension of urn cell complexes in equal volumes of 0.01 M concentrations of any of the eight free-thiol compounds listed below for 5 min before adding a strong stock stimulus induced urn cell complexes to produce very long, periodic compact tails within 2–5 min (Fig. 9 inset). This was in contrast to the cloudy, precipitate-covered tails induced in the same sample of urn cell complexes by the same stmulus without pre-incubation in the free-thiol compounds (Fig. 9). At no time was there solubilization of the secreted mucus. Nine S-H compounds that altered and enhanced secretion produced by urn cell complexes following stimulus by heated serum were L-cysteine, D-cysteine, N-acetyl-L-cysteine, 1,4-dithio-L-threitol (L-DTT), dithioerythretol (DTE), S-carboxymethyl-L-cysteine, 2-mercapto-ethanol, thioglycolic acid, and glutathione.

Also tested were 6 amino acids without free S-H terminals on the molecule. Incubation of urn cell complexes in each of these before addition of the stock stimulus produced no change in the morphology of the tail, nor was there any effect



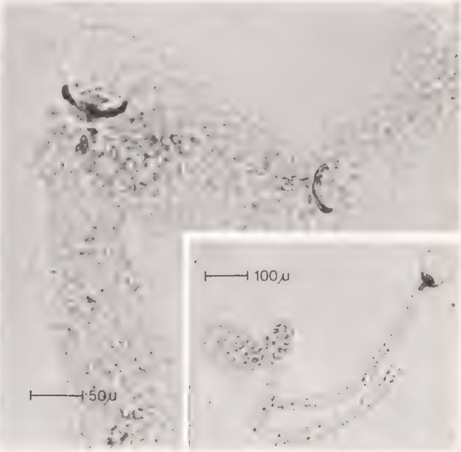


FIGURE 8. (Left) Living urn cell complexes were stimulated with mucus-stimulating human serum, then heat-killed *S. nudus* erythrocytes were added. These were treated as non-self and were scavenged by urn cell complexes. Red cells accumulated most avidly at R cell level.

FIGURE 9. (Right) Living urn cell complexes photographed 10 min after exposure to mucus-stimulating human serum: copious secretory tails covered with background precipitate. Inset: Living urn cell complex from same culture incubated 5 min in glutathione, then exposed to same stimulus and photographed in 10 min: long, thin, periodic, compact secretory tail.

on the secreted mucus. The following 12 amino acids failed to enhance or alter secretion: L-cystine, L-cysteic acid, L-alanine, N-acetyl-L-alanine, L-phenylalanine, L-asparagine, L-arginine, L-glutamic acid, L-proline, L-serine, and L-methionine.

The function of the secretory systems in nature. There is no way of knowing the ages of individual *S. nudus* that are sources of supernatant urn cell complexes. Nor can one judge the spectrum of "ages" (*i.e.*, the time after release from the epithelial lining) of individual complexes within a supernatant population. Also unknown is the recent history of exposure of each *S. nudus* to various types of stress in its habitat. For these reasons, laboratory-maintained *S. nudus* cannot be expected to provide urn cell complexes which give statistically standard responses, a variable which must be taken into account in any comparative study. It is advisable to use urn cell complexes from the same supernatant preparation for any comparative study. At least 500 tests can be carried out with equal portions of supernatant from 2 to 3 ml of whole *S. nudus* blood.

We tried to gain some insight into the amount of stimulus to which the secretory systems might be routinely exposed in nature. Thirty animals were bled in the the field under sterile conditions. Then, after a 45-min drive back to the laboratory, the supernatant urn cell complexes of each of the 30 test-tube preparations were examined microscopically for "spontaneous" hypersecretion. Urn cell complexes in 13, or 43%, of the preparations were producing small tails of varying lengths and shapes, usually a clear granular secretion. Agar plate cultures of a drop of blood from each of these same animals had been prepared in the field, and after 3 days' culture at room temperature, there were no visible bacterial colonies. Thus, the spectrum of infectious agents or toxins which induce urn cell complexes to produce secretory tails of differing qualities in nature remains to be determined.

Discussion

Our previous papers on responses of urn cell complexes to stimuli have emphasized effects on the locus system, since it is this system which responds differently to body fluids of humans and rabbits in normal and dseased states. It is now clear, however, that the effects of R cells and their products on the nature of the final (combined) urn cell complex secretion may be crucial in *S. nudus in vivo*. There are two points to be made about the biological activity of R cells. One is that *in vivo* trapping of activated and dead cells by R cells is the first stage in progressive phagocytosis of this cell debris by autologous leukocytes. We suggest therefore that R cells may themselves be phagocytic: like vertebrate macrophages, they rapidly and avidly pinocytose neutral red, and vertebrate physiologists now recognize that the classical macrophage is a secretory cell (Unanue *et al.*, 1976). Dybas (1976) described the phagocytic properties of the free urn cell complex of another Sipunculid, *Phascolosoma agassizii*, a complex comprising three constituent cell types, two of which are selectively phagocytic.

The second point is that understanding the relationship between R cell secretory activity and the nature of locus secretion may be very useful in understanding the nature and control of analogous secretory responses in human diseases that affect mucous secretory systems. In human respiratory mucous membranes, for example, there is continuous interaction between serous and mucous gland cells. The surface mucus derives from mixed mucous-serous acini and from goblet cells, ontogenically different cell systems (Tos, 1976; Bang, 1964). Qualitative changes in the surface mucus may result from airborne stimuli (allergens or irritants), from infectious agents, or from systemic defects. Cystic fibrosis exemplifies the latter: the respiratory mucous membranes of cystic fibrosis patients function normally *until* bacterial infection sets in; normal individuals recover mucociliary function with no adverse sequelae, while cystic fibrosis patients are unable to produce mucus of normal consistency after such an infection; tenacious mucus is anchored in the gland cells and produces varying degrees of mucociliary stasis.

In living specimens of *S. Nudus*, during acute bacterial infections all urn cell complexes are engaged in producing mucus which immobilizes, traps, and probably lyses the bacteria. This race between bacterial replication and urn cell complex activity ends either with death of the *S. nudus* or elimination of the bacteria. During low-grade infections, there is a clear advantage to having the hypersecretory (locus) system controlled by the R cells: enough mucus is produced to trap the bacteria without energy-expensive hypersecretion of large volumes of mucus. During acute infection, however, bacterial products themselves may cleave the R cells from the base cell and allow uncontrolled hypersecretion to proceed to eliminate the bacteria. The data suggest that R cells and loci each have receptors for stimuli that have particular molecular conformations.

The nature of the stimuli and of the receptors, the composition of R cell secretion and locus secretions, the ultrastructure of the respective secretory systems, and the role of the fluid space enclosed by the vesicle and the base cell all remain to be investigated. Radio isotope labeling of metabolic precursors and of the active components of defined stimuli may begin to resolve the biochemical questions; electron microscopy should identify the secretory apparatuses. The urn cell complex provides a system in which the regulation and production of mucus can be systematically explored *in vitro*, and a biological system in which mucus stimulating substances in invertebrate and vertebrate body fluids can be comparatively assayed in health and disease.

ACKNOWLEDGMENTS

The flowing-nitrogen chamber was kindly loaned to us at Roscoff by Dr. Manfred Grieshaber of the Zoologisches Institut der Westfälischen Wilhelms-Universität, Münster, West Germany.

We thank Dr. Moon L. Shin, Associate Professor of Pathology, University of Maryland School of Medicine, for her innovative contributions to the model, and the administrative and field staffs of the Station Biologique, Roscoff, for their continued cooperation.

LITERATURE CITED

BANG, B. G., 1964. The mucous glands of the developing human nose. *Acta Anat.*, **59**: 297–314.

BANG, B. G., AND F. B. BANG, 1965. Mucus hypersecretion in a normally isolated non-innervated cell. *Cah. Biol. Mar.*, **6**: 257–264.

BANG, B. G., AND F. B. BANG, 1976. The mucous secretory apparatus of the free urn cell of *Sipunculus nudus*. *Cah. Biol. Mar.*, **17**: 423–432.

BANG, B. G., AND F. B. BANG, 1979. Mucus-stimulating substances in human body fluids assayed in an invertebrate mucous cell system. *Johns Hopkins Med. J.*, **145**: 209–216.

BANG, F. B., 1966. Serologic response in a marine worm, *Sipunculus nudus*. *J. Immunol.*, **96**: 960–972.

BANG, F. B., AND B. G. BANG, 1962. Studies on sipunculid blood: immunologic properties of coelomic fluid and morphology of "urn cells." *Cah. Biol. Mar.*, **3**: 363–374.

BEREITER-HAHN, J., 1976. Dimethylaminostyrylmethylpyridinium-iodine (DASPMI) as a fluorescent probe for mitochondria *in situ*. *Biochim. Biophys. Acta*, **423**: 1–14.

DYBAS, L., 1976. A light and electron microscopic study of the ciliated urn of *Phascolosoma agassizii* (Sipunculida). *Cell Tissue Res.*, **169**: 67–75.

LAZAROW, A., AND S. J. COOPERSTEIN, 1953. Studies on the mechanism of Janus green B staining of mitochondria. *Exp. Cell Res.*, **5**: 56–97.

REISSIG, M., B. G. BANG, AND F. B. BANG, 1979. Mucus secretion in the urn complex of *Sipunculus nudus*. *J. Cell Biol.*, **83**: SP2512 (abstract).

TOS, M., 1976. Mucous elements in the nose. *Rhinology*, **14**: 155–162.

UNANUE, E. R., D. I. BELLER, J. CALDERON, J. M. KIELY, AND M. J. STADECKER, 1976. Regulation of immunity and inflammation by mediators from macrophages. *Am. J. Pathol.*, **85**: 466–478.

Reference: *Biol. Bull.*, **159**: 582–591. (December, 1980)

ULTRASTRUCTURAL CHARACTERISTICS OF THE NON-EXPANDED AND EXPANDED EXTRA-EMBRYONIC SHELL OF THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* L.

GARY A. BANNON[1] AND GEORGE GORDON BROWN

Department of Zoology, Iowa State University, Ames, Iowa 50011 and
Hopkins Marine Station of Stanford University, Pacific Grove, California 93950

ABSTRACT

During embryonic development of the horseshoe crab, *Limulus polyphemus,* a structural layer, the extra-embryonic shell (EES), develops in the periembryonic space. Late in development the egg envelope breaks away and the EES expands slowly (over a period of several days) to approximately twice its original diameter. The EES is composed of non-cellular material organized into three distinct layers. The outermost (layer 1) is electron translucent and exhibits numerous hairlike projections. The middle layer (layer 2) is differentiated from layer 1 by its greater density under the electron beam. The innermost layer (layer 3) comprises 99% of the mass of the EES and is intermediate in electron density between layers 1 and 2. The inside surface of the non-expanded EES is smooth, and easily distinguished from the outside surface, which is rough and accentuated by polygonal structures. The deep indentations separating these structures are probable sources of preformed surface area utilized during EES expansion. The expansion of the EES occurs concomitantly with cracking of the egg envelope when the embryo has reached stage 20. As expansion continues, the distance between the once tightly packed polygonal structures is greatly increased. However, the thickness of the expanded shell is less than expected if expansion of the EES is only the result of utilization of surface area stored in the indentations. Therefore, EES expansion appears to be made possible by at least two mechanisms: (1) Utilization of preformed surface area stored in the indentation, and (2) Stretching of the shell—implying that the EES exhibits limited elasticity during development. Expansion of the EES is necessary to provide the space required for later larval development.

INTRODUCTION

The envelopes of developing embryos are important for protection from mechanical injury, maintenance of a constant environmental medium, and passage of respiratory gases (Wigglesworth, 1946). These envelopes, depending on their origin, have been classified as primary, secondary, or tertiary (Quattropani and Anderson, 1969; Ludwig, 1874). Primary envelopes are produced by the oocyte, secondary by ovarian follicle cells, and tertiary by an extra-ovarian source (*e.g.*, the developing embryo) (Quattropani and Anderson, 1969). The formation, structure, and function of primary and secondary envelopes have been described

Abbreviations: EES, extra-embryonic shell; ASW, artificial seawater.

Received August 10, 1980; accepted September 25, 1980.

[1] All communication should be with this author.

for a number of invertebrates (Regier *et al.*, 1980; Mazur *et al.*, 1980; Turner and Mahowald, 1976; Barbier and Chauvin, 1974; Salkeld, 1973; Quattropani and Anderson, 1969; Cheung, 1966). The tertiary envelope, however, has been less thoroughly examined, probably because many organisms do not form a true envelope of this origin (*e.g.*, the fertilization membrane of sea urchins can be described as one of primary and tertiary origins because it is formed from a combination of the oocyte-produced vitelline layer and materials excreted by the embryo during the cortical reaction; Chandler and Heuser, 1980; Boyan, 1970a, b).

In the American horseshoe crab, *Limulus polyphemus,* all three types of envelopes are found. Eggs possess an egg envelope consisting of an inner vitelline envelope of primary origin and an outer basement lamina of secondary origin. The formation and structure of these layers have been previously described (Shoger and Brown, 1970; Dumont and Anderson, 1967). However, in *Limulus* and the closely related Japanese horseshoe crab, *Tachypleus tridentatus,* the formation and structure of the extra-embryonic shell (a true tertiary envelope) has been examined only superficially (Kingsley, 1892; Sekiguchi, 1970).

The extra-embryonic shell (EES) appears in the periembryonic space during early development of the *Limulus* embryo and is completed prior to stage 17 (Brown and Clapper, 1980). During stage 20 (approximately 21 days after insemination at 20°–21°C) the egg envelope cracks off and the EES slowly expands to approximately twice its previous diameter. In this expanded sphere, the larva is active, and several days after the cracking of the egg envelope moults into a trilobite larva (stage 21). Eventually, the trilobite larva will hatch from the EES and become free-swimming. Similar observations have also been recorded by Sekiguchi (1973) and Sugita and Sekiguchi (1979) on *Tachypleus tridentatus.*

This report describes the organization of the extra-embryonic shell (EES) before and after expansion. Possible mechanisms by which the EES expands are also discussed.

MATERIALS AND METHODS

Source of Animals

Specimens of *Limulus polyphemus* L. (obtained from the Florida Marine Biological Specimen Co., Inc., Panama City, Florida) were maintained at 15°C in Instant Ocean Aquaria in artificial sea water (ASW) (Aquarium Systems, Inc., Eastlake, Ohio) on a 14:10 L:D photoperiod.

Gamete collection and insemination

Gametes were collected by brief electrical stimulation (1–1.5 V, 0.5–1.0 mA, ac) of the gonoducts proximal to the gonopores (Brown and Clapper, 1980). Semen was diluted with 100% ASW (960 m-osmoles) to produce a 10% sperm suspension (10^9 spermatozoa/ml). Eggs (40–50) were placed in a plastic petri dish containing 25 ml of ASW. Two drops of the sperm suspension were added and the mixture was gently swirled. All cultures were maintained at room temperature (20°–21°C).

Procurement of embryos before and after expansion of EES

Specimens representing different steps of expansion of the EES were collected for electron microscopic preparation before, during, and after cracking of the egg

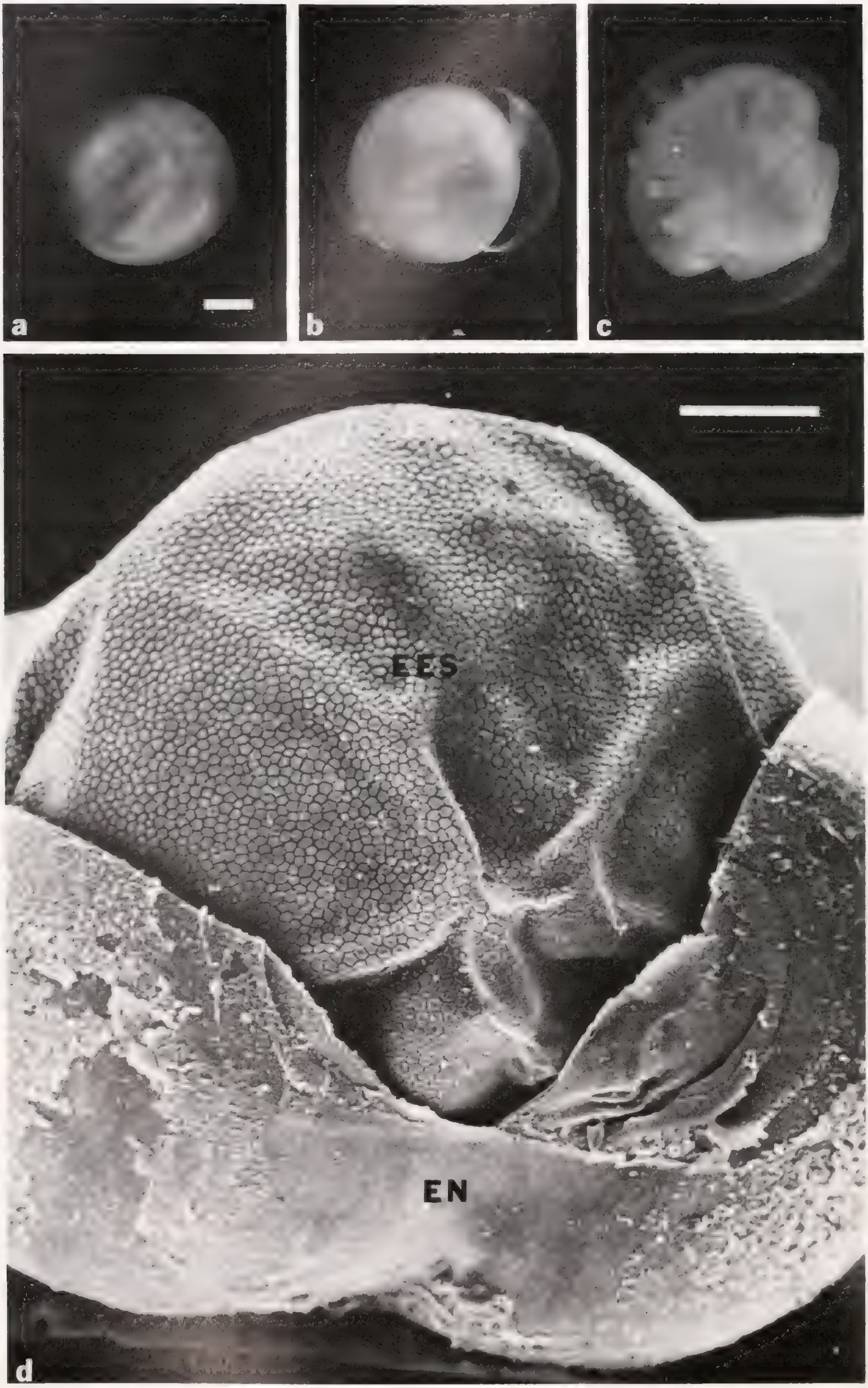


FIGURE 1. Expansion of the extra-embryonic shell: (a) The embryo is enclosed by the extra-embryonic shell and the egg envelope. Bar = 1.0 mm. (b) The embryo has moulted

envelope and subsequent expansion of the EES. The first step was obtained when embryos were in stage 19 (approximately 19 days after fertilization). The second step was obtained after stage 20 appeared and cracking of the egg envelope was in process. Specimens for the third step were usually collected several days after cracking had occurred. The expansion process was measured daily until hatching and was recorded photographically using a Wild M-5 dissecting microscope.

Electron microscopy

Properly staged specimens were fixed in a 2.5% glutaraldehyde solution (cacodylate buffer; 4°C) for 12–24 hr, post-fixed in 2% osmium tetroxide and stored in double distilled H_2O for one or more days. For critical point drying, utilizing liquid carbon dioxide, fixed specimens were dehydrated in ethanol and cleared into amylacetate. Some dried specimens were fractured with a sharp razor blade. For freeze-drying, fixed specimens were snap-frozen, one at a time, in liquid nitrogen, placed on pre-cooled brass blocks, and evacuated (5×10^{-5} Torr) overnight. All specimens were mounted on stubs with silver paint, carbon and gold coated, and examined on a Cambridge Mark 2A "Stereoscan" scanning electron microscope.

Similarly staged specimens, to be examined by transmission electron microscopy, were fixed in a trialdehyde solution (Kalt and Tandler, 1971; acrolein, 0.178 M; DMSO, 0.32 M; formaldehyde, 0.66 M; glutaraldehyde, 0.33 M: pH, 7.0) for 3 hr, post-fixed in 1% osmium tetroxide and dehydrated. All fixation procedures were carried out at 4°C. The sample was infiltrated by puncturing the embryo with a sharp probe before embedding it in Spurrs resin (Standard Medium A; Polysciences Data Sheet No. 127). All tissue was sectioned on a LKB Ultrotome, stained with aqueous uranyl acetate and lead citrate and examined on a Hitachi HU-11E-1 electron microscope.

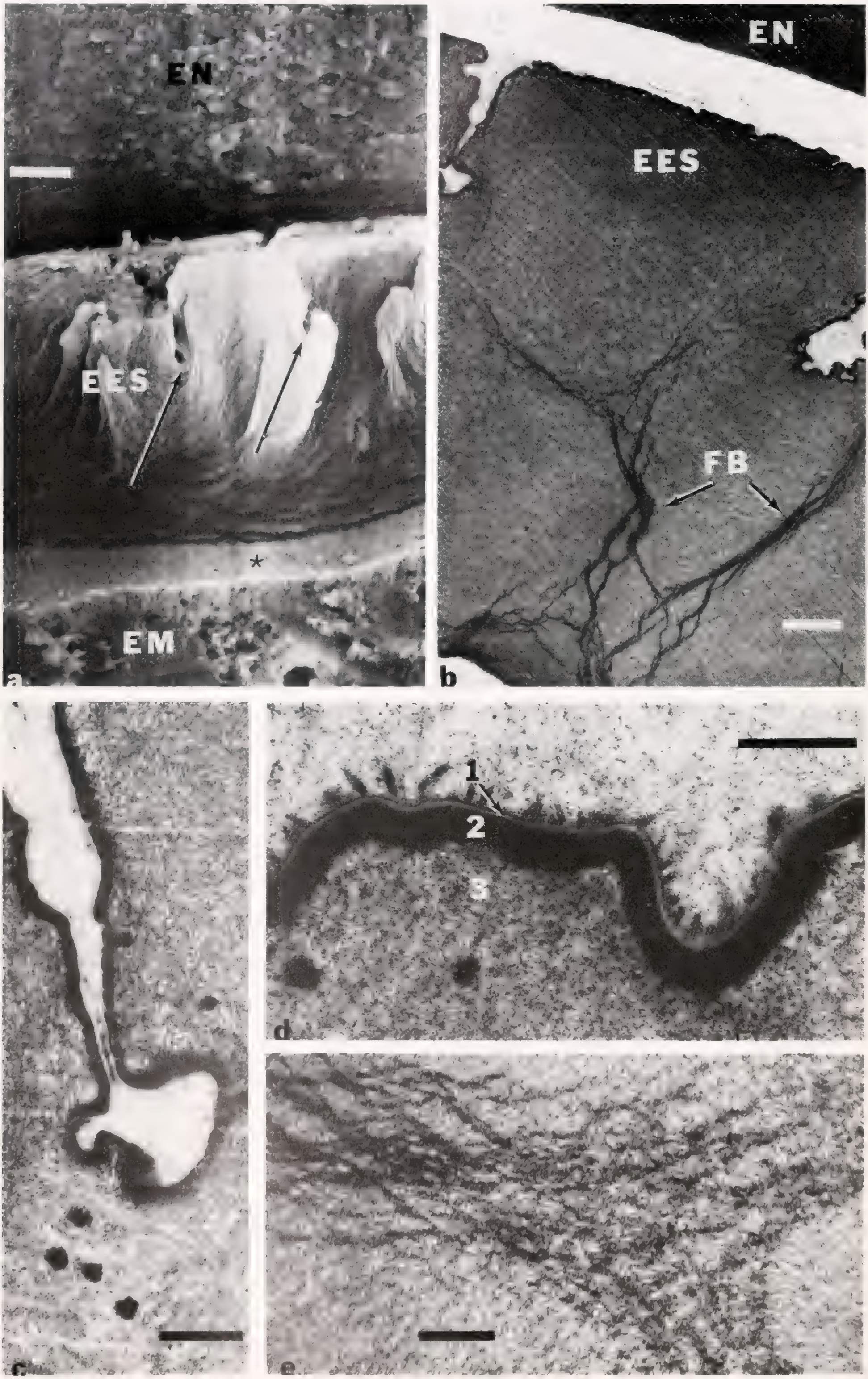
RESULTS

General observations

Approximately 14 days elapse from the first step of the expansion process (Figs. 1a–c) to hatching (19th–33rd day after fertilization). The diameter of the enclosing envelopes during this process increases from an initial 3.8 to 6.4 mm at hatching. This increase in diameter of the EES is very slow during expansion, even immediately after sloughing of the egg envelope (Fig. 1b). The EES forms in the periembryonic space between the egg envelope and embryo proper and appears to be morphologically complete by stage 14 (limb-bud stage, 12 days after fertilization). Obvious polygonal structures can be observed easily after the egg envelope cracks (Fig. 1d).

---

into stage 20 and the egg envelope is cracking away. The extra-embryonic shell is exposed and is slowly expanding. Full expansion takes several days. Same scale as (a). (c) The fully expanded extra-embryonic shell allows an increased space in which the stage 20 embryo can be active and undergo the final embryonic moult into the trilobite larva (stage 21). Same scale as (a). (d) Scanning electron micrograph (SEM) of the cracking of the egg envelope (EN). The extra-embryonic shell (EES) is characterized by the presence of numerous packed polygonal structures which gives the surface a rough, irregular appearance. Bar = 0.5 mm.


EN
EES
*
EM
a
EN
EES
FB
b
c
1
2
3
d
e

Extra-embryonic shell before expansion

The EES before expansion is evident in a section through a stage 19 embryo (Fig. 2a). Also apparent are the egg envelope and the developing embryo. The egg envelope consists of two morphologically distinct layers, a 5-μm-thick basement lamina and a 40-μm-thick vitelline envelope (Shoger and Brown, 1970; Dumont and Anderson, 1967). The EES is approximately 33.16 μm thick and consists of swirls of non-cellular materials with the outer third containing regularly spaced indentations. Closely apposed to the inside surface of the EES is the chitinous exoskeleton of the embryo.

The EES is composed of three distinct layers (Figs. 2b, d). The outermost (layer 1) is approximately 0.02 μm thick, electron translucent (Fig. 2d), and the source of numerous hairlike projections. The middle layer (layer 2) is approximately 0.14 μm thick and electron dense. Layer 3, the innermost, comprises the majority of mass of the EES and is approximately 33 μm thick and intermediate in electron density between layers 1 and 2. Fibrous bundles are observed throughout layer 3 (Figs. 2b, e). Associated with the indentations observed in the EES in Figures 2a and b are electron-dense bodies (Fig. 2c).

Extra-embryonic shell after expansion

After expansion the exterior surface of the EES consists of numerous variably-shaped polygonal structures separated by a large interpolygonal region with a very irregular surface (Fig. 3a). As the EES expands this region is formed by elevation and flattening of the previously described indentations between the polygonal structures.

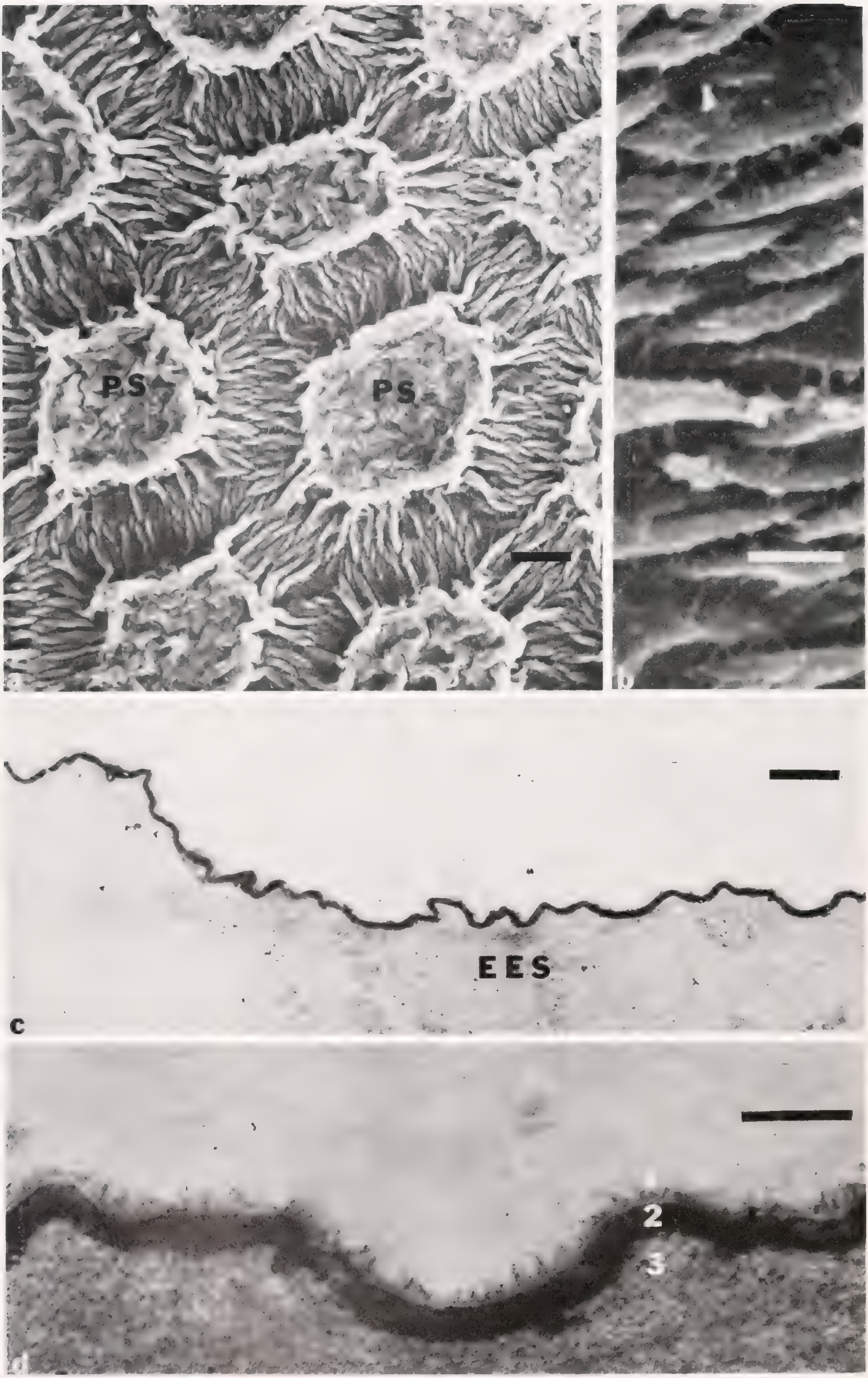
On the surface, the interpolygonal region appears to be composed of many ridges or "fibers" radiating from each polygonal structure, and interdigitating with "fibers" from other polygonal structures (Fig. 3a). This arrangement can also be observed on an unfixed expanded EES with light microscopy. Between these "fibers" hairlike projections give the appearance of cross-linking (Fig. 3b). Cross sections reveal the surface is very irregular and these "fibers" are ridges on the outer surface of the EES (Fig. 3c, d). The thickness of the fully expanded shell (measured at widest portion) is reduced to 12 μm, a 64% reduction from the non-expanded shell. The three layers which comprise the EES retain the characteristics seen in the non-expanded form, except for the absence of fibrous bundles and electron-dense bodies in layer 3 (Figs. 3c, d).

DISCUSSION

For xiphosurid embryology, the use of the term extra-embryonic shell (EES) is recommended since this structure is formed outside the embryo, is composed of noncellular material, and is formed by embryonic secretions which may occur over

---

FIGURE 2. Electron microscopy of non-expanded shell: (a) SEM of fractured stage-19 embryo and surrounding coats. The EES develops between the egg envelope (EN) and the chitinous exoskeleton (*) of the embryo (EM) in the periembryonic space. Indentations in the EES are clearly visible (arrows). Bar = 5 μm. (b) TEM of non-expanded EES showing large indentations and fibrous bundles (FB). Bar = 3 μm. (c) Longitudinal section of an indentation in the non-expanded shell. Dense bodies are associated with the indentations. Bar = 1 μm. (d) Cross-section of the non-expanded EES detailing the three layers which compose this structure. Bar = 0.5 μm. (e) Fibrous bundles of layer 3 in non-expanded EES. Bar = 0.5 μm.


PS
PS
a
b
c
EES
2
3
d

several developmental stages. Other terms have been used: blastoderm cuticle (Roonwal, 1944), blastodermhaut (Kingsley, 1892), deutovum (Iwanoff, 1933), inner egg membrane (Sekiguchi, 1970), and egg membrane (French, 1979). These are inappropriate because of the above characteristics.

However, the EES of *Limulus* has some similarities to a typical insect cuticle. An insect cuticle is constructed asymmetrically with the outermost layer representing the first layer produced by the epidermis and subsequent layers distinguished by their different electron densities as observed with electron microscopy (*cf.* Neville, 1975). The insect cuticle may be composed of numerous layers (Caveny, 1971). The EES is also asymmetrical but consists of only three layers, which could correspond, at least spatially, to the epicuticle, exocuticle, and endocuticle of insect cuticle. The EES differs from a typical insect cuticle in its relative simplicity and its separation from any cellular layer by a periembryonic space.

The expansion of the EES involves utilization of preformed surface area stored in the large indentations observed in the non-expanded shell (Fig. 2b). However, the size increase of the totally expanded shell requires more surface area than found in these indentations. For example, before expansion, the thickness of the EES from the bottom of the indentations to the inside surface of the EES is 22 μm. But when the expanded shell reaches maximum size, its width is only 12 μm at the widest point, indicating that the noncellular components of the EES must be rearranged (stretched) to compensate for the decreased thickness of the expanded shell. As measured in this study and others (Sekiguchi, 1970; Roonwal, 1944), the EES continues a slow expansion until hatching of the larva.

The mechanism for expansion has been considered by various investigators (Kingsley, 1892; Iwanoff, 1933; Roonwal, 1944; Sugita and Sekiguchi, 1979). Several theories have been presented. A favorite one involves osmotic effectors produced by the embryo to allow an increase in fluid content in the periembryonic space. However, little supporting evidence has been provided. Recently, using *Tachypleus*, Sugita and Sekiguchi (1979) have examined the complement of proteins present in the periembryonic ("perivitelline") space and described four classes of proteins, H, B-1, B-2, and "rest." Changes in concentration of these proteins were followed during development. The "rest" proteins increased dramatically before expansion of the EES, indicating their possible involvement in this process.

According to Yamamichi and Sekiguchi (1974) and Sugita and Sekiguchi (1979) the EES is formed from material secreted into the periembryonic space by blastoderm cells. The timetable for secretion of these components into the perivitelline space and the mechanisms of transport are not known. However, it is possible that some of the components reach the perivitelline space via the egg cortical reaction (Bannon and Brown, 1980). The question now being addressed in our laboratory is whether these components are synthesized during

FIGURE 3. Electron microscopy of expanded shell: (a) SEM of exterior surface of expanded EES showing polygonal structures (PS) and the large interpolygonal region with interdigitating "fibers." Bar = 5 μm. (b) SEM of interpolygonal region. This region is very irregular and numerous hairlike projections can be seen originating from the "fibers." Bar = 1 μm. (c) TEM of expanded shell. The large indentations, fibrous bundles, and electron dense bodies are no longer present. Bar = 2 μm. (d) Cross section of expanded EES showing that the basic structure of the shell does not change after expansion (1, layer 1; 2, layer 2; 3, layer 3). (*Cf.* Fig. 2d.) The irregular surface topography represents the "fibers" observed in 3a. Bar = 0.5 μm.

oogenesis and stored in the early embryo until needed, or synthesized by the embryo during development.

ACKNOWLEDGMENTS

The authors acknowledge the use of Dr. Walt Humphreys' SEM facilities at the Department of Biology, University of Georgia, and give special thanks to Dr. George Dearlove, Department of Biology, Hofstra University, Hempstead, New York, for his critical reading of the manuscript. This research was supported in part by the Iowa State University Graduate College, an Iowa State University Research Grant, an Iowa State University Faculty Improvement leave, and a gift from Houston Endowment, Inc.

LITERATURE CITED

BANNON, G. A., AND G. G. BROWN, 1980. Vesicle involvement in the egg cortical reaction of the horseshoe crab, *Limulus polyphemus* L. *Dev. Biol.*, **76**: 418–427.

BARBIER, R., AND G. CHAUVIN, 1974. The aquatic egg of *Nymphula nymphaeata* (Lepidoptera: Pyralidae). On the fine structure of the egg shell. *Cell Tiss. Res.*, **149**: 473–479.

BOYAN, J., 1970a. The isolation of a major structural element of the sea urchin fertilization membrane. *J. Cell Biol.*, **44**: 635–644.

BOYAN, J., 1970b. On the reconstitution of the crystalline components of the sea urchin fertilization membrane. *J. Cell Biol.*, **45**: 606–614.

BROWN, G. G., AND D. L. CLAPPER, in press. Procedures for collecting gametes and culturing embryos of the horseshoe crab, *Limulus polyphemus*. In Ralph Hinegardner *et al.*, Eds., *Marine Invertebrates, Laboratory Animal Maintenance*. National Academy of Science, Washington, D. C.

CAVENY, S., 1971. Control of Cuticle Architecture in Insects. Ph.D. thesis, Oxford Univ., England.

CHANDLER, D. E., AND J. HEUSER, 1980. The vitelline layer of the sea urchin egg and its modification during fertilization. *J. Cell Biol.*, **84**: 618–632.

CHEUNG, T. S., 1966. The development of egg membranes and egg attachment in the shore crab, *Carcinus maenus* and some related decapods. *J. Mar. Biol. Assn. U. K.*, **46**: 373–400.

DUMONT, J. N., AND E. ANDERSON, 1967. Vitellogenesis in the horseshoe crab, *Limulus polyphemus*. *J. Microsc.*, **6**: 791–806.

FRENCH, K. A., 1979. Laboratory culture of embryonic and juvenile *Limulus*. Pp. 61–71 in *Biomedical Applications of the Horseshoe Crab (Limulida)*. Alan R. Liss, Inc., New York.

IWANOFF, P. P., 1933. Die embryonale Entwicklung von *Limulus moluccanis*. *Zool. Jahrb.*, **56**: 164–346.

KALT, M. R., AND B. TANDLER, 1971. A study of fixation of early amphibian embryos for electron microscopy. *J. Ultrastruct. Res.*, **36**: 633–645.

KINGSLEY, J. S., 1892. The Embryology of *Limulus*. *J. Morphol.*, **7**: 35–68.

LUDWIG, H., 1874. Uber die Eibildung in Thierreiche. *Wuzb Zool. Inst. Arb.*, **1**: 287–510.

MAZUR, G. D., J. C. REGIER, AND F. C. KAFATOS, 1980. The silkmoth chorion: Morphogenesis of surface structures and its relation to synthesis of specific proteins. *Dev. Biol.*, **76**: 305–321.

NEVILLE, A. C., 1975. *Biology of the Arthropod Cuticle*. Springer Verlag, *Berlin*. 448 pages.

QUATTROPANI, S. L., AND E. ANDERSON, 1969. The origin and structure of the secondary coat of the egg of *Drosophila melanogaster*. *Z. Zellforsch.*, **85**: 495–510.

REGIER, J. C., G. D. MAZUR, AND F. C. KAFATOS, 1980. The silkmoth chorion: Morphological and biochemical characterization of four surface regions. *Dev. Biol.*, **76**: 286–304.

ROONWAL, M. L., 1944. Some observations on the breeding biology and on the swelling, weight, water content and embryonic movement in the developing eggs of the Moluccan king crab, *Tachypleus gigas* (Müller). *Proc. Indian Acad. Sci. Sec. B*, **20**: 115–129.

SALKELD, E. H., 1973. The chorionic architecture and shell structure of Amathes *C-Nigrum* (Lepidoptera: Noctuidae). *Can. Entomol.*, **105**: 1–10.

SEKIGUCHI, K., 1970. On the inner egg membrane of the horseshoe crab. *Zool. Mag. (Tokyo)*, **79**: 115–118.

SEKIGUCHI, K., 1973. A normal plate of the development of the Japanese horseshoe crab, *Tachypleus tridentatus*. *Sci. Rep. Tokyo Kyoiku Daigaku Sect. B*, **15**: 153–162.

SHOGER, R. L., AND G. G. BROWN, 1970. Ultrastructural study of sperm-egg inteactions of the horseshoe crab, *Limulus polyphemus* L. (Merostomata: Xiphosura). *J. Submicrosc. Cytol.*, **2**: 167–179

SUGITA, H., AND K. SEKIGUCHI, 1979. Protein components in the perivitelline fluid of the embryo of the horseshoe crab, *Tachypleus tridentatus*. *Dev. Biol.*, **73**: 183–192.

TURNER, F. R., AND A. P. MAHOWALD, 1976. Scanning electron microscopy of *Drosophila* embryogenesis: 1. The structure of the egg envelopes and the formation of the cellular blastoderm. *Dev. Biol.* **50**: 95–108.

WIGGLESWORTH, V. B., 1946. *The Principles of Insect Physiology*. Chapman and Hall, London. Pp. 1–26.

YAMAMICHI, Y., AND K. SEKIGUCHI, 1974. Embryo and organ cultures of the horseshoe crab, *Tachypleus tridentatus*. *Dev. Growth Differ.*, **16**: 295–304.

Reference: *Biol. Bull.*, **159**: 592–605. (December, 1980)

THE LARVAL DEVELOPMENT OF *PINNIXA LONGIPES* (LOCKINGTON, 1877) (BRACHYURA: PINNOTHERIDAE), REARED IN THE LABORATORY

GEORGE D. BOUSQUETTE[1]

Pacific Marine Station, Dillon Beach, California, and University of the Pacific, Stockton, California

ABSTRACT

Larvae of *Pinnixa longipes*, a commensal with the polychaete *Axiothella rubrocincta*, were reared to the megalopa stage in the laboratory. The zoeae were cultured at 34‰, 20°C, fed a combination of *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* daily, and provided with a photoperiod of 12 h each of light and dark. The five zoeal and one megalopal stages are described and figured in detail, including the arrangement of chromatophores and various types of setae. Zoeae of *P. longipes* can be distinguished from other Pinnotherid zoeae, for which descriptions are available, on the basis of morphological characteristics.

INTRODUCTION

The pea crab, *Pinnixa longipes* (Lockington, 1877), is commensal with the tube building polychaete, *Axiothella rubrocincta* (Johnson, 1901). This worm constructs U-shaped tubes in sand-mud substrata of bays and estuaries along the Pacific coast (Kudenov, 1977). The pronounced lateral elongation and diminutive size of *P. longipes* enables it to move easily in the narrow tube with the worm. The species has occasionally been found with other polychaetes, including *Pectinaria* and *Pista* (Carlton and Kuris, 1975). According to Schmitt *et al.* (1973), *Pinnixa longipes* ranges from Tomales Bay to Laguna Beach, California. There are no published accounts regarding the biology of this commensal crab.

The present paper describes in detail the larval development of *P. longipes* reared under laboratory conditions. These results are compared with previous descriptions of pinnotherid larvae.

MATERIALS AND METHODS

Ovigerous specimens of *Pinnixa longipes* were collected on 13 and 18 November 1978 from *Axiothella rubrocincta* tubes at White Gulch in Tomales Bay, California. In the laboratory, the ovigerous females were placed in finger bowls (10-cm diameter) with filtered seawater at 34‰ salinity. This salinity was maintained by mixing Instant Ocean or glass-distilled water with filtered seawater. A constant temperature of 20°C was maintained in a refrigerator supplied with fluorescent lights to provide 12 h each of light and dark. Adult females were not fed during the experiment. The filtered seawater was changed daily and the bowls inspected for larvae.

Received September 9, 1979; accepted August 20, 1980.

[1] Current address: 2480 I St., Petaluma, CA 94952.

After hatching, the larvae were placed in finger bowls (10-cm diameter) and maintained under the same salinity, temperature, and light parameters as the adults. From 10 to 20 zoeae were put in each bowl . Each day, after changing the medium, approximately 2 ml each of concentrated *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* were added to the bowls. The algae were cultured in a half-strength enriched seawater medium "f" (Guillard and Ryther, 1962). During the experiment, algal material was clearly evident within the larval digestive tracts. (Preliminary culturing observations in the early summer of 1978 demonstrated that *Artemia salina* is an inappropriate food for *P. longipes* larvae.)

Larvae of each stage were preserved in ethylene glycol along with the dead zoeae and exuviae. Pigmentation was observed on both living and preserved specimens. Drawings were made from preserved larvae and exuviae using a camera lucida, calibrated with a stage micrometer. Appendages were dissected with fine needles. Descriptions and illustrations were checked against at least five specimens from each stage of development.

RESULTS

Pigmentation

The location of chromatophores is fairly consistent throughout the zoeal development of *Pinnixa longipes*. The arrangement of chromatophores is diagramed in Figure 1. The eyes are dark reddish brown in color. A pair of yellow chromatophores is located on abdominal segment 5 near its attachment to the telson, and a similar pair is found on the posterior-lateral portion of abdominal segment 4 (Fig. 1 A and C, 1 and 2 respectively). A single yellow chromatophore is present on abdominal segments 2 and 3 in a posterior-medial position (Fig. 1A and C, 3 and 4). On the first abdominal segment a yellow chromatophore is situated anteriorly and medially (Figs. 1A and C, 5).

A yellow chromatophore is located on the distal portion of the basipodite of the first maxilliped (Fig. 1A and B, 6), and also on the mandible (Fig. 1A, 7). One

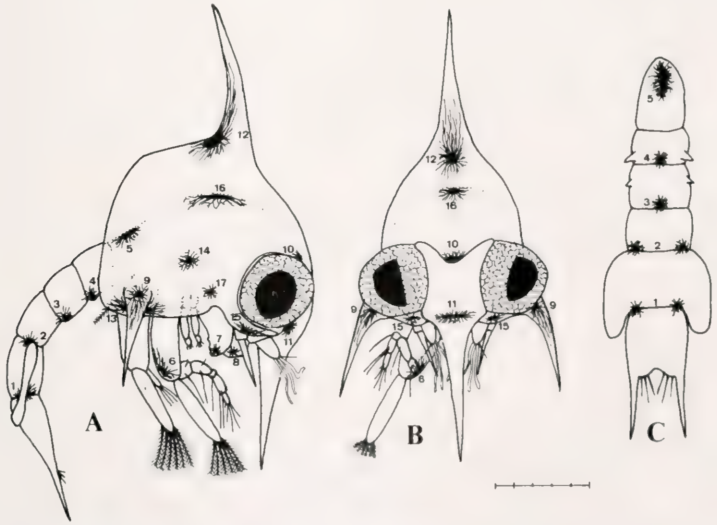


FIGURE 1. Diagrammatic sketches of arrangement of chromatophores on Zoea II of *Pinnixa longipes*: A, side view; B, frontal view of cephalothorax; C, ventral view of abdomen.

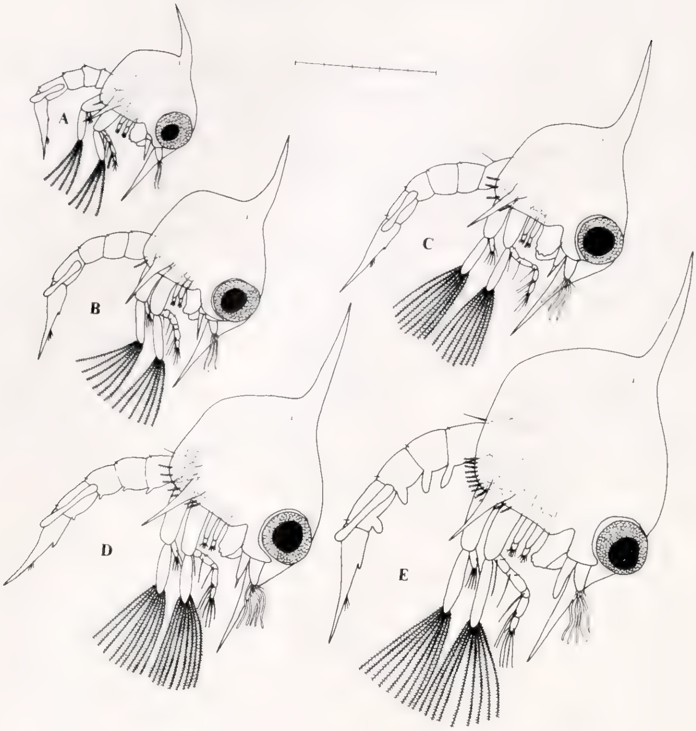


FIGURE 2. Lateral view of zoeae of *Pinnixa longipes*: A, Zoea I; B, Zoea II; C, Zoea III; D, Zoea IV; E, Zoea V. Scale is 0.5 mm.

dark red chromatophore is evident on the labrum (Fig. 1A, 8). Various chromatophores are found on the carapace itself. A large red one appears at the base of each lateral spine (Fig. 1A, B, 9), between the eyes dorsally and ventrally (Fig. 1A and B, 10 and 11), and at the base of the dorsal spine in a posterior position (Fig. 1A and B, 12). Also, single large yellow chromatophores occur along the posterior-ventral margin of the carapace below each lateral spine (Fig. 1A, 13), dorsal and anterior to each lateral spine (Fig. 1A, 14), and near the base of each antennule-antenna set (Fig. 1A and B, 15).

A large yellow chromatophore is found above the heart and stomach (Fig. 1A and B, 16), while a red chromatophore occurs near the base of each mandible (Fig. 1A, 17).

Description of larval stages

Five zoeal stages and one megalopa were observed. The duration of each intermolt period was as follows: Zoea I, 6–7 days, Zoea II, 4–5 days, Zoea III, 4–6 days, Zoea IV, 6–8 days, Zoea V, 6–9 Days. After 8 days the megalopa had exhibited no further molting. The average time required to reach the megalopal stage was 26.75 days ($n = 4$, s.d. $= 0.9574$). The major morphological character-

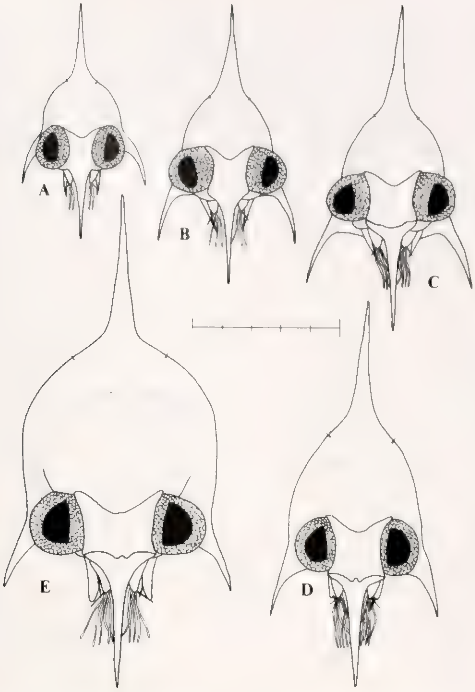


FIGURE 3. Frontal view of the carapace of *Pinnixa longipes* zoeae: A, Zoea I; B, Zoea II; C, Zoea III; D, Zoea IV; E, Zoea V. Scale is 0.5 mm.

istics of each developmental stage are detailed below:

Zoea I (Fig. 2A, 3A). Dorsal, rostral, and lateral spines are well developed. Minute plumose setae arise between the dorsal and lateral spines (Fig. 2A, 3A). The eyes are sessile. The abdomen (Fig. 4A) is composed of a telson and five segments. Segments 2 and 3 have rounded lobes projecting laterally. The fifth segment has lateral extensions which overlap the telson. Each furcal ramus of the telson bears three serrate setae on its inner margin. The medial and lateral margins of each ramus possesses a row of very fine acuminate setae. A thickening overlies the anal opening.

The antennule is conical in outline with two long aesthetascs and one short acuminate seta located terminally (Fig. 5A).

The antenna has an elongated protopodite with two opposing rows of minute serrations on its distal half. From the proximal portion of the protopodite an acuminate seta projects laterally (Fig. 5F).

The mandibles are symmetrical with two incisors located ventrally and a molar process located dorsally (Fig. 6A).

Very fine acuminate setae are found on the proximal margin of the coxal and basal endites of the maxillule (Fig. 5K) and the maxilla (Fig. 5P). On the maxilla, these setae are also found on each side of the endopod, and entirely fringing the scaphognathite except where plumose setae are located.

The first and second maxilliped are pictured in Figures 7A and 8A respectively, while the third maxilliped has not developed.

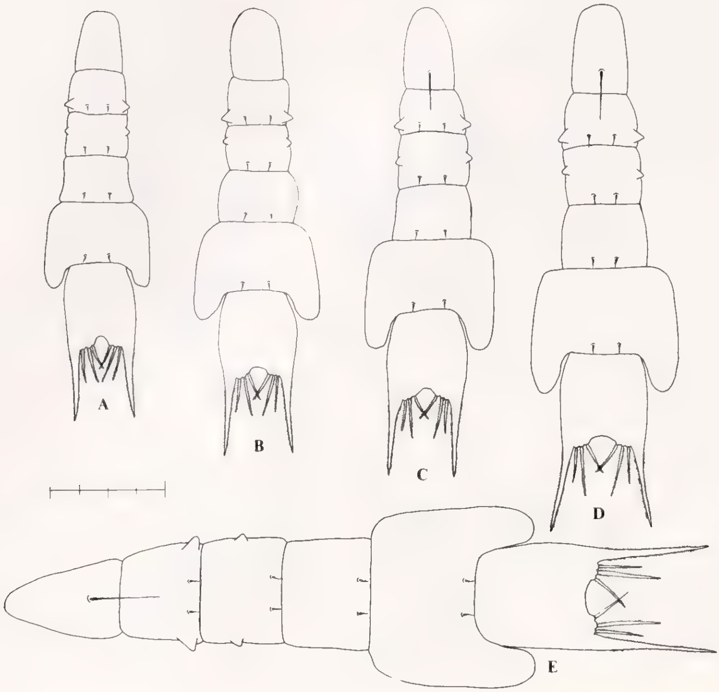


FIGURE 4. Dorsal view of the abdomen of *Pinnixa longipes* zoeae: A, Zoea I; B, Zoea II; C, Zoea III; D, Zoea IV; E, Zoea V. Scale is 0.2 mm.

Zoea II (Fig. 2B, 3B). The characteristics of the cephalothorax and abdomen are similar to Zoea I except that the eyes are now stalked. Figure 5 illustrates the changes in form and setation which have occurred on the antennule, antenna, maxillule, and the maxilla.

The setal arrangements found on the first and second maxillipeds are shown in Figures 7 and 8, respectively. The third maxilliped has not yet developed.

Zoea III (Fig. 2C, 3C). The cephalothorax is similar to the preceding stage except that there are now four plumose setae on the posterior-ventral margin of the carapace. A long, acuminate seta arises dorsally from the distal portion of the first abdominal segment (Fig. 4C).

A bulge occurring at the base of the serrated portion of the protopodite of the antenna represents the endopodite bud (Fig. 5H). The third maxillipeds are still undeveloped.

Zoea IV (Fig. 2D, 3D). Seven plumose setae are now evident on the posterior-ventral margin of the carapace. Pleopod buds are visible for the first time, with a pair found on each of segments 2–5.

Another incisor has been added to the two already present ventrally and a fourth is now evident on the same plane, but is more dorsal in position (Fig. 6D). A medial extension of the coxa is evident on the second maxilliped of this stage (Fig. 8D).

Rounded buds of the third maxilliped and pereiopods are beneath the carapace (Fig. 2D). The claw of the first pereiopod is clearly bifurcated, as is the third maxilliped (Fig. 9A).

Zoea V (Fig. 2E, 3E). The cephalothorax is similar to that in the preceding stage except that 12 plumose setae are now present on the posterior-ventral margin of the carapace. The pleopod buds have increased in size (Fig. 9B).

The mandible now has five teeth present on one plane, three ventral and two dorsal, while the more dorsally located molar plane has acquired four teeth at its margins (Fig. 6E).

The third maxilliped and pereiopod buds have greatly increased in size (Fig. 9C). Indications of segmentation are visible in the third maxilliped and the pereiopods.

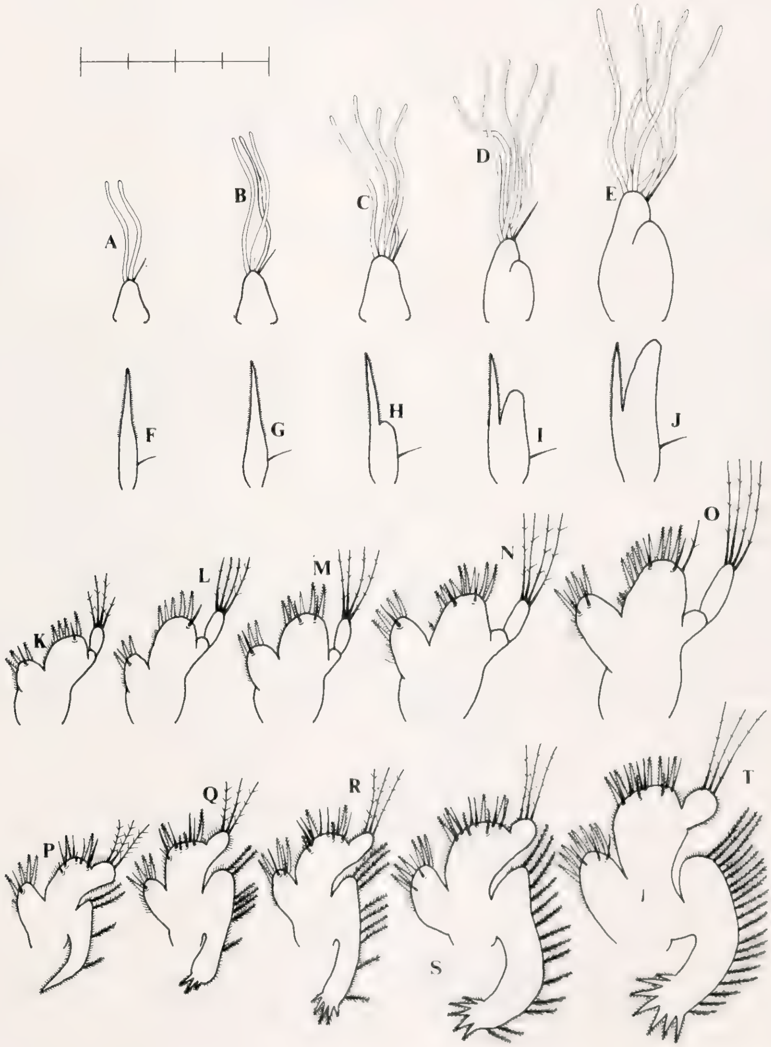


FIGURE 5. Antennules, antennae, maxillules, and maxillae of *Pinnixa longipes* zoeae: A–E, antennules of Zoea I–V; F–J, antennae of Zoea I–V; K–O, maxillules of Zoea I–V; P–T, maxillae of Zoea I–V. Scale is 0.2 mm.

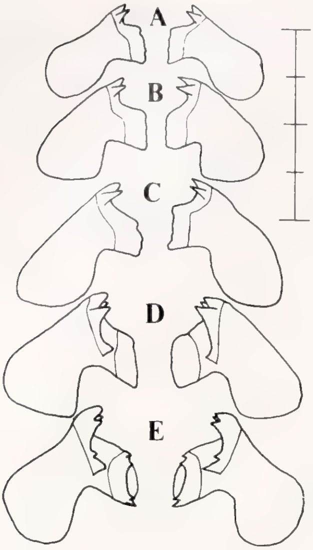


FIGURE 6. Mandibles of *Pinnixa longipes* zoeae: A, Zoea I; B, Zoea II; C, Zoea III; D, Zoea IV; E, Zoea V. Scale is 0.2 mm.

Megalopa (Fig. 10A–C, 11). The carapace of *P. longipes* is 1½ times wider than it is long, with a squared rostrum separating the stalked eyes. The dorsal surface of the carapace is smooth except for the presence of 42 minute, acuminate setae and one small plumose seta just posterior and lateral to each eye (Fig. 10A).

7

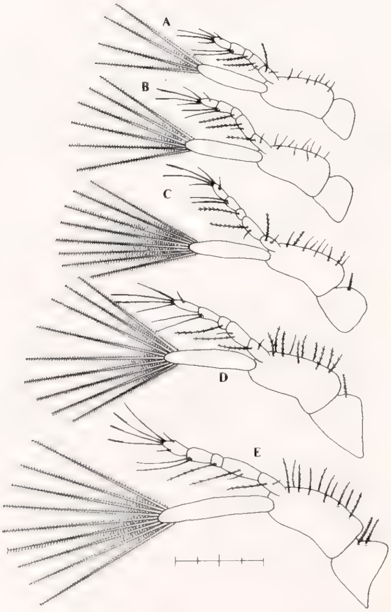


FIGURE 7. (Left) First maxillipeds of *Pinnixa longipes* zoeae: A. Zoea I; B, Zoea II; C, Zoea III; D, Zoea IV; E, Zoea V. Scale is 0.2 mm.

8

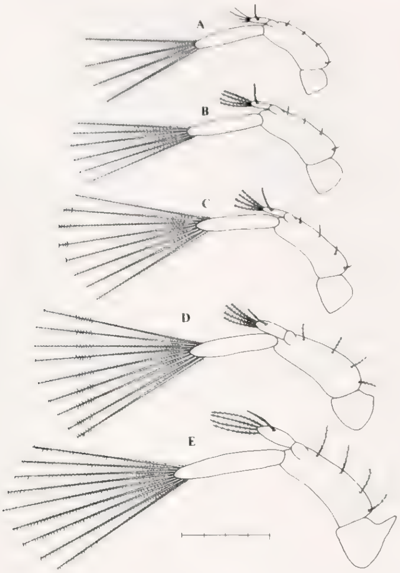


FIGURE 8. (Right) Second maxillipeds of *Pinnixa longipes* zoeae: A, Zoea I; B, Zoea II; C, Zoea III; D, Zoea IV; E, Zoea V. Scale is 0.2 mm.

Twenty-five plumose setae are in evidence along the length of the ventral border of the carapace starting near the cheliped and extending to the fourth pereiopod (not pictured).

The mandible consists of a cutting edge with five teeth and a basal palp which bears 14 small, plumose setae terminally and a longer one subterminally (Fig. 10D).

The very fine acuminate setae found on the maxillule and maxilla of the zoeal stages are no longer evident.

The dactylus of the first pereiopod has a medial lobe facing the longer fixed finger of the propodus, which has a subterminal notch into which the tip of the dactylus fits (Fig. 10A). Proceeding from the second pereiopod to the fifth they become more paddle-like. The fourth pereiopod bears two small, rounded lumps on the posterior margin of the ischium, and seven more each on the posterior and anterior margins of the merus. Two similar protuberances are located on the posterior margin of the ischium of the fifth pereiopod along with a single one on its

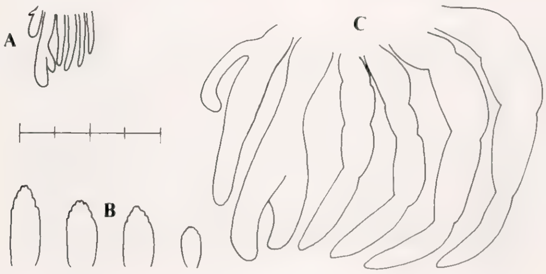


FIGURE 9. Third maxillipeds, pereiopods, and pleopod buds found on *Pinnixa longipes* zoeae: A, buds of third maxilliped and pereiopods on Zoea IV; B, pleopod buds on Zoea V; C, third maxilliped and pereiopod buds on Zoea V. Scale is 0.2 mm.

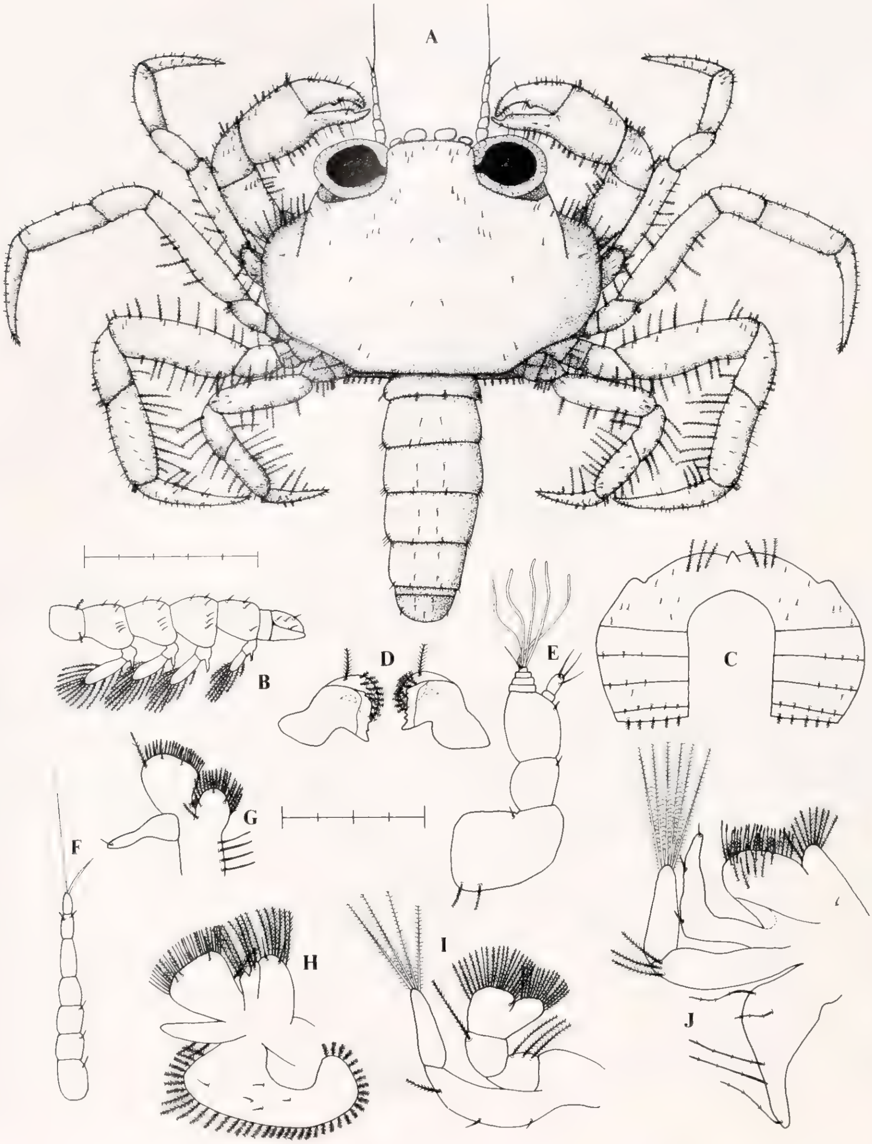


FIGURE 10. Megalopa of *Pinnixa longipes*: A, dorsal view; B, lateral view of abdomen; C, thoracic sternum; D, mandibles; E, antennule; F, antenna; G, maxillule; H, maxilla; I, second maxilliped; J, first maxilliped. Scale is 0.5 mm for A–C, and 0.2 mm for D–J.

anterior margin. Two more are found on the posterior margin of the merus of the fifth pereiopod.

The pleopods are found on abdominal segments 2-5 (Fig. 12A–D). Two hook setae are located terminally on the endopod of each pleopod.

DISCUSSION

Pinnotherid crabs have attracted considerable attention through the years, primarily due to their highly modified and varied commensal relationships with

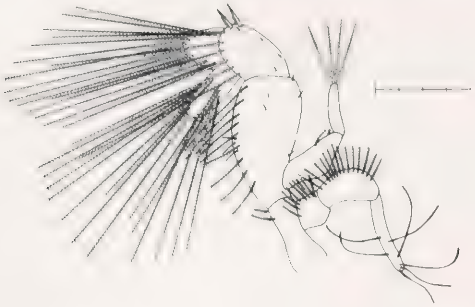


FIGURE 11. Third maxilliped of *Pinnixa longipes* megalopa. Scale is 0.2 mm.

other invertebrates. In recent years, the need for accurate identification of planktonic larval forms has been recognized by ecologists undertaking population and community research. Unfortunately, except for a few species infesting hosts of commercial importance, there have been few investigations of the larval development of pinnotherids. Larval descriptions are presently available for 24 species in nine genera within the Pinnotheridae (Table I). Of these species, the complete larval development is known for only 11 species, 2 of which belong to *Pinnixa.*

Based upon morphological characters little difficulty is encountered in separating the zoeae of *Pinnixa* from the other Pinnotherid genera. *P. longipes* exhibits the unique fifth abdominal segment which Hyman (1924) reports to be characteristic for the genus. The lateral and posterior extension of this segment was noted for *P. chaetopterana* and *P. sayana* by Faxon (1879), and for *P. rathbuni* by Sekiguchi (1978). It is evident from the drawings and description supplied by Irvine and Coffin (1960) that *Fabia subquadrata* also possesses this special abdominal modification, and is dissimilar in only a few characteristics from *P. longipes.*

The zoeae of *P. longipes* have two setae on the first two segments of the endopodite of the first maxilliped, and a small acuminate seta on the antennule in addition to the aesthetascs. On the other hand, *Fabia subquadrata*, which has been reported from Alaska to San Diego, California (Schmitt *et al.*, 1973), has only one seta on the first two segments of the endopodite of the first maxilliped, and no acuminate seta on the antennule. In addition, Irvine and Coffin (1960) report a humped condition in zoeae of *F. subquadrata.* They pictured this as lateral ridges on the carapace extending between the eyes and dorsal spine.

Faxon (1879) and Hyman (1924) described the first zoeal stage of *P. chaetopterana*, whose range extends from Massachusetts to Brazil (Schmitt *et al.*, 1973), making its occurrence with *P. longipes* unlikely. One particular character,

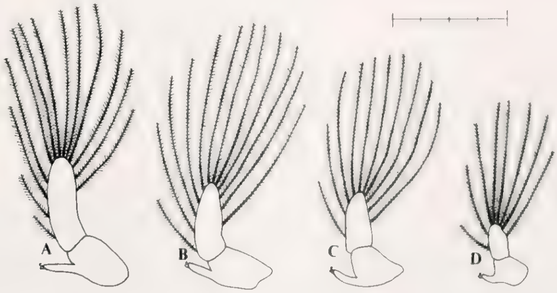


FIGURE 12. Pleopods of *Pinnixa longipes* megalopa: A, first pleopod; B, second pleopod; C, third pleopod; D, fourth pleopod. Scale is 0.2 mm.

TABLE I

Survey of available larval descriptions within the family Pinnotheridae.

Genus	Species	Author	Stage/s described[1]
Dissodactylus	*mellitae*	Hyman, 1924	2
Fabia	*subquadrata*	Irvine and Coffin, 1960	1
Ostracotheres	*tridacnae*	Gurney, 1938	2
		Gohar and Al-Kholy, 1957	1
Parapinnixa	*affinis*	Glassell, 1933	2
Pinnaxodes	*chilensis*	Schwabe, 1936	2
		Guiterrez-Martinez, 1971	2
	major	Hong, 1974	1
Pinnotheres	*chamae*	Roberts, 1975	1
	hickmani	Pregenzer, 1979	2
	holothuriae	Semper, 1881	11
		Hyman, 1924 (after Semper)	10
	latissimus	Miyake, 1935	3
	maculatus	Faxon, 1879	9
		Hyman, 1924	2
		Costlow and Bookhout, 1966	1
	moseri	Goodbody, 1960	10
	novaezelandiae	Bennett, 1964	2
		Pregenzer, 1979	2
	ostreum	Birge, 1882	2
		Hyman, 1924	3
		Sandoz and Hopkins, 1947	8
	pinnotheres	Gourret, 1882	9
		Lebour, 1928	1
		Gurney, 1942	2
		Atkins, 1955, 1960	1
Pinnotheres	*pisum*	Thompson, 1835	11
		Lebour, 1928	5
		Atkins, 1955, 1960	1
		Rice, 1975	2
	placunae	Hashmi, 1970	4
	ridgewayi	Prasad and Tampi, 1957	2
	taylori	Hart, 1935	1
	sp.	Menon, 1937	6
Pinnixa	*chaetopterana*	Faxon, 1879	2
		Smith, 1880	2
		Hyman, 1924	2
	longipes	Bousquette, 1979	1
	rathbuni	Sekiguchi, 1978	1
		Muroaka, 1979	7
	sayana	Faxon, 1879	7
Asthenognathus	*atlanticus*	Bocquet, 1965	1

---

[1] Key to stage/s described: 1 = All zoeal stages and megalopa described. 2 = Stage I described. 3 = Stage I and II described. 4 = Stage I and III described. 5 = Stage I (and possibly II) described. 6 = Stage II and last zoeal stage described. 7 = Last zoeal stage and megalopa described. 8 = All zoeal stages and megalopa described (stage IV partial). 9 = Telson of indeterminable stage described. 10 = General description of zoeal stage I. 11 = Stage I pictured.

however, makes it quite easy to separate these two species. *P. chaetopterana* has a large deltoid tooth projecting medially from the furcal arch. *P. longipes* lacks this feature.

The last zoeal stage of *P. sayana* as described by Faxon (1879) is difficult to separate from *P. longipes*, but its range from Massachusetts to Louisiana plus Brazil precludes its occurrence with *P. longipes*. However, in comparison, the only apparent difference is the presence of two terminal setae on the endopodite of the maxilla of *P. sayana* and three in *P. longipes*.

The zoeae of *P. rathbuni*, whose geographic distribution extends from southeastern Siberia to Japanese waters, exhibit dorsal and rostral spines which are approximately twice as long as the carapace (Sekiguchi, 1978), whereas in *P. longipes* these spines about equal the carapace in length.

Descriptions of the megalopa are available for 12 pinnotherids. *P. longipes* can be distinguished from the majority of these species by its greater carapace width than length. The megalopa of *P. sayana* and *P. rathbuni* also exhibit this characteristic, but *P. sayana* has a row of small spines on the sides of the carapace (Faxon, 1879), while *P. rathbuni* has a set of large spines on each anterior-lateral margin of the carapace. The borders of the carapace of *P. longipes* are smooth.

In their work regarding larval descriptions within the subfamily Portuninae, Bookhout and Costlow (1974, 1977) noted the need for more detailed accounts of morphological characteristics due to the high degree to which members of this group resemble each other. They found that measurements of different body structures and setal armature of appendages are useful in distinguishing between similar species. A set of tables listing particular body structure measurements, and detailed descriptions of setal types found on the various appendages of *P. longipes* may be found in Bousquette (1979).

Presently, the descriptions available are sufficient to differentiate between described pinnotherid species in most instances, but in the future as we study closely related larval forms this ability will most likely decrease unless we adopt the format of Bookhout and Costlow (1974, 1977). Concurrently, further complete larval descriptions will increase our understanding of taxonomic groupings now found in the Pinnotheridae, where need is evidenced by the great similarity between zoeal forms of *Fabia subquadrata* and *Pinnixa longipes*.

Acknowledgments

I'm grateful to Dr. James Blake for his encouragement, critical review, and help with the preparation of this manuscript. I also would like to thank Vickey L. Quinn-Bousquette and Francis R. Gens for their aid in the field and laboratory during the course of this study.

This work represents part of a thesis submitted in partial fulfillment for the Master of Science degree at the University of the Pacific. The actual project was conducted at the Pacific Marine Station, Dillon Beach, California.

LITERATURE CITED

Atkins, D., 1955. The post-embryonic development of British *Pinnotheres* (Crustacea). *Proc. Zool. Soc. Lond.*, **124**: 687–715.

Atkins, D., 1960. The development of the pleopods in the young crab stages of *Pinnotheres* (Crustacea). *Proc. Zool. Soc. Lond.*, **133**: 435–451.

Bennett, E. W., 1964. The marine fauna of New Zealand; Crustacea, Brachyura. *N. Z. Bull. Sci. Indust. Res.*, **153**: 1–120.

BIRGE, E. A., 1882. General notes on the first zoea stages of *Pinnotheres ostreum*. *Am. Nat.*, **16**: 589–591.

BOCQUET, CHARLES, 1965. Stades larvaires et juvéniles de *Tritodynamia atlantica* (Th. Monod) (= *Asthenognathus atlanticus* Th. Monod) et position systématique de ce crabe. *Cah. Biol. Mar.*, **6**: 407–418.

BOOKHOUT, C. G., AND JOHN D. COSTLOW, JR., 1974. Larval development of *Portunus spinicarpus* reared in the laboratory. *Bull. Mar. Sci.*, **24**: 20–51.

BOOKHOUT, C. G., AND JOHN D. COSTLOW, JR., 1977. Larval development of *Callinectes similis* reared in the laboratory. *Bull. Mar. Sci.*, **27**: 704–728.

BOUSQUETTE, G. D., 1979. The larval development of *Pinnixa longipes* (Lockington, 1877) (Brachyura: Pinnotheridae), reared in the laboratory. Masters Thesis at the University of the Pacific, Stockton, California. 53 pp.

CARLTON, JAMES T., AND ARMAND M. KURIS, 1975. Keys to decapod crustacea. Pp. 385–412 in R. I. Smith and J. T. Carlton, Eds., *Light's manual: Intertidal invertebrates of the central California coast.* University of California Press, Berkeley, Los Angeles, London.

COSTLOW, JOHN D., JR., AND C. G. BOOKHOUT, 1966. Larval stages of the crab, *Pinnotheres maculatus*, under laboratory conditions. *Chesapeake Sci.*, **7**: 157–163.

FAXON, WALTER, 1879. On some young stages in the development of *Hippa-Porcellana* and *Pinnixa*. *Bull. Mus. Comp. Zool.*, **5**: 253–268.

GLASSELL, STEVE A., 1933. Notes on *Parapinnixa affinis* Holmes and its allies. *Trans. San Diego Soc. Nat. Hist.*, **7**: 319–330.

GOHAR, H. A. R., AND A. A. AL-KHOLY, 1957. The larvae of four decapod crustacea (from the Red Sea). *Publ. Mar. Biol. Sta. Al Ghardaqa*, **9**: 153–160.

GOODBODY, I., 1960. Abbreviated development in a Pinnotherid crab. *Nature*, **185**: 704–705.

GOURRETT, P., 1882. Considérations sur la faune pélagique du golfe de Marseille. *Ann. Mus. Hist. Nat. Marseille (Zool.)*, **2**: 14–24.

GUILLARD, R. R. L., AND J. H. RYTHER, 1962. Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve). *Can. J. Microbiol.*, **8**: 229–239.

GURNEY, ROBERT, 1938. Notes on some decapod crustacea from the Red Sea, VI–VIII. VI: Some brachyuran larvae. *Proc. Zool. Soc. Lond.*, **108**: 73–84.

GURNEY, ROBERT, 1942. *Larvae of decapod crustacea.* Ray Society, London, 306 pp.

GUTIERREZ-MARTINEZ, JUAN, 1971. Notas biológicas sobre *Pinnaxodes chilensis* (M. Edwards) y descripción de su primera Zoea (Crustacea Decapoda, Brachyura). *Mus. Nac. Hist. Nat. Mens., Santiago*, **15**: 3–10.

HART, J., 1935. The larval development of British Columbia brachyura. I. Xanthidae, Pinnotheridae (in part) and Grapsidae. *Can. J. Res.*, **12**: 419–432.

HASHMI, SYED SALAHUDDIN, 1970. The larvae of *Elamena* (Hymenosomidae) and *Pinnotheres* (Pinnotheridae) hatched in the laboratory (Decapod: Crustacea). *Pak. J. Sci. Indust. Res.*, **12**: 279–285.

HONG, SUNG YUN, 1974. The larval development of *Pinnaxodes major* Ortman (Decapoda, Brachyura, Pinnotheridae) under the laboratory conditions. *Publ. Mar. Lab. Pusan Fish. Coll.*, **7**: 87–99.

HYMAN, O. W., 1924. Studies on larvae of crabs of the family Pinnotheridae. *Proc. U. S. Nat. Mus.*, **64**: 1–9.

IRVINE, JOHN, AND HAROLD G. COFFIN, 1960. Laboratory culture and early stages of *Fabia subquadrata* (Dana), (Crustacea, Decapoda). *Walla Walla Coll. Publ.*, No. 28, 24 pp.

KUDENOV, J., 1977. The functional morphology of feeding in three species of maldanid polychaetes. *Zool. J. Linn. Soc.*, **60**: 95–109.

LEBOUR, M. V., 1928. Studies of the Plymouth Brachyura. II. The larval stages of *Ebalia* and *Pinnotheres*. *J. Mar. Biol. Assoc. U. K.*, **15**: 109–124.

MENON, M. K., 1937. Decapod larvae from the Madras plankton. *Bull. Madras Govt. Mus. (Natur Hist. Sect.)*, **3**: 1–55.

MIYAKE, SADAYOSHI, 1935. Note on the zoeal stages of *Pinnotheres latissimus*. *Bulteno Scienca de la Fakultato Terkultura Kjusu Imperia Univ. Fukuoka, Japanujo*, **6**: 192–201.

MURAOKA, KENSAKU, 1979. Post-larva of *Pinnixa rathbuni* (Crustacea, Brachyura, Pinnotheridae). *Zool. Mag., Tokyo*, **88**: 288–294.

PRASAD, R. R., AND P. R. S. TAMPI, 1957. Notes on some decapod larvae. *J. Zool. Soc. India*, **9**: 22–39.

PREGENZER, C., JR., 1979. A redescription of *Pinnotheres hickmani* new combination and comparison with *Pinnotheres pisum* (Decapoda, Brachyura). *Crustaceana Suppl. (Lieden)*, **0** (Suppl. 5): 22–30.

RICE, A. L., 1975. The first zoeal stages of *Cancer pagurus* L., *Pinnotheres pisum* (Pennant) and *Macrophthalmus depressus* Ruppell (Crustacea, Decapoda, Brachyura). *Bull. Br. Mus. (Nat. Hist.) Zool.*, **28**: 237–247.

ROBERTS, MORRIS H., JR., 1975. Larval development of *Pinnotheres chamae* reared in the laboratory. *Chesapeake Sci.*, **16**: 242–252.

SANDOZ, MILDRED, AND SEWELL H. HOPKINS, 1947. Early life history of the oyster crab, *Pinnotheres ostreum* (Say). *Biol. Bull.*, **93**: 250–258.

SCHMITT, WALDO L., JOHN C. MCCAIN, AND EDWARD S. DAVIDSON, 1973. Decapoda I. Brachyura I. Family Pinnotheridae. 160 pp. in H. E. Gruner and L. B. Holthuis, Eds., *Crustaceorum Catalogus*. Den Haag, W. Junk (publ.), 160 pp.

SCHWABE, G. H., 1936. Investigaciones sobre *Loxechinus albus* Mol. y *Pinnotheres chilensis* Edw. *Bol. Soc. Biol. Concepcion*, **10**: 125–136.

SEKIGUCHI, H., 1978. Larvae of a pinnotherid crab, *Pinnixa rathbuni* Sakai. *Proc. Jap. Soc. Syst. Zool.*, **15**: 36–46.

SEMPER, CARL, 1881. *Animal life as affected by the natural conditions of existence*. D. Appleton and Company, New York, 472 pp.

SMITH, S. I., 1880. On the species of *Pinnixa* inhabiting the New England coast, with remarks on their early stages. *Trans. Conn. Acad. Sci.*, **4**: 247–253.

THOMPSON, J. V., 1835. Memoirs on the metamorphosis and natural history of the *Pinnotheres* or pea-crabs. *Lond. Entomol. Mag.*, **3**: 85–90.

Reference: *Biol. Bull.*, **159**: 606–612. (December, 1980)

AGE DETERMINATION IN THE KEYHOLE LIMPET *FISSURELLA CRASSA* LAMARCK (ARCHAEOGASTROPODA: FISSURELLIDAE), BASED ON SHELL GROWTH RINGS

MARTA BRETOS

Centro de Investigaciones Marinas, Universidad del Norte Sede Iquique,
Casilla 65, Iquique, Chile

ABSTRACT

Field studies at Huayquique, northern Chile, using tagged specimens of *Fissurella crassa* released in their natural habitat, found that shell-growth rings were formed twice a year, usually in summer and winter. Age-size relationships, based on mean growth ring sizes, were established by using the von Bertalanffy growth equation:

$$L_t = 94.5148\ (1 - e^{-0.1590(t+1.9999)}).$$

Shell sizes at different ages were compared with the growth pattern previously determined for this species. Growth rings were shown to be a reliable method for determining age in *Fissurella crassa* at Huayquique, at least in animals from 1–6 years old. The largest specimen analyzed had a calculated age of 10 years.

INTRODUCTION

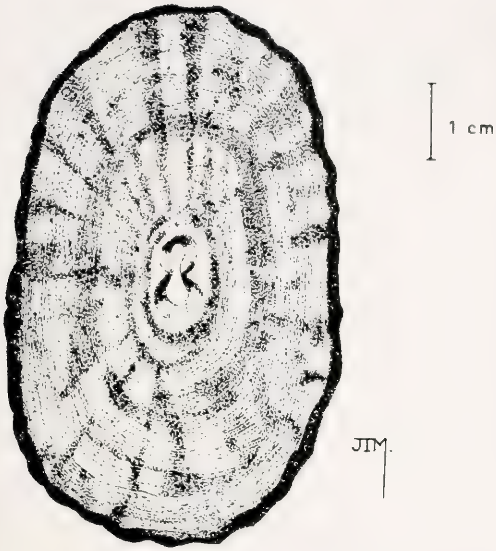
An outstanding feature of molluscan growth is its variation in rate (Wilbur and Owen, 1964). Nevertheless, growth and age in molluscs can be determined using several methods, one of which is the study of growth rings (Sakai, 1960; Newman, 1968; Jones *et al.*, 1978).

Variations in molluscan growth rate through the life span often result in concentric shell markings known as growth rings. These formations are a result of changes in mantle secretory activity (Wilbur and Owen, 1964).

Several conditions may bring about ring formation: (1) Rings may appear regularly in certain seasons, for example, winter or summer rings resulting from inhibited growth at low or high temperatures (Wilbur and Owen, 1964). (2) Disturbance rings may be formed as a result of changes in environmental conditions, changes in diet (Sakai, 1960), handling (Coe, 1947), marking of animals (Jones *et al.*, 1978), or similar disturbances. (3) A reproductive period may induce ring formation (spawning rings) due to temporary decrease or interruption of growth (Morton, 1969; Jones *et al.*, 1978; Brousseau, 1979; Salzwedel, 1979). (In addition, narrow subdaily growth striations formed by concentrations of organic material, and revealed by scanning electron micrographs, correspond in width to the amount of shell material that would be expected to dissolve during periods of anaerobics metabolism of *Mercenaria mercenaria*; Gordon and Carriker, 1978.)

The reliability of growth rings as a basis for determining growth and age depends mainly on the regularity of ring formation in the species under study.

Received August 5, 1980; accepted September 15, 1980.



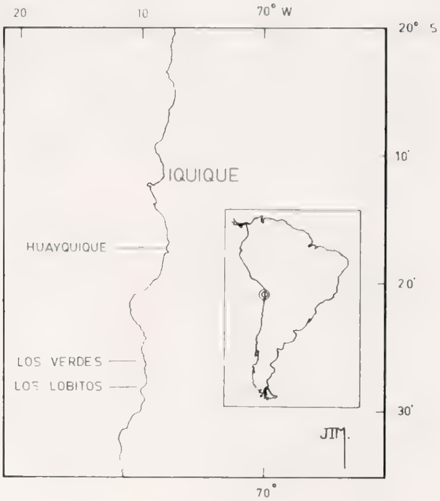


FIGURE 1. (Left) Shell of a *Fissurella crassa* specimen from Huayquique.
FIGURE 2. (Right) Research locations.

Previous experimental research on growth of the keyhole limpet *Fissurella crassa* Lamarck, 1822 (Fig. 1) in northern Chile has revealed that during two periods in the year growth ceases or decreases (Bretos, 1978) suggesting that this species might form two growth rings per year. The present investigation was undertaken to determine how many growth rings *Fissurella crassa* formed per year in northern Chile, the periodicity of formation, and the validity of using growth rings for age determination in this species.

MATERIALS AND METHODS

Three groups of *Fissurella crassa* were analyzed in this study: one of marked animals maintained in their natural habitat, and two of unmarked controls, not disturbed by tagging, measuring, or recapture.

Experimental group

The periodicity of growth ring formation was tested by using individually marked *Fissurella crassa* specimens. The experiment was carried out on the rocky shore of Huayquique, northern Chile (20° 17′ S) (Fig. 2), in front of the Centro de Investigaciones Marinas, from October 1975 to September 1978.

A total of 496 animals caught in the experimental intertidal area were tagged, measured, and released. Tagged specimens were recaptured and measured after being gently removed from the substrate, usually once a month during spring tides.

Two types of marks were used to identify each experimental animal: (a) small numbered tags glued to the clean, dry shell and covered with Dekophane adhesive; (b) serial notches sawed near the apical orifice and on the shell margin. The second type of mark was usually preferred because it persisted longer and was easily detected in recaptured animals (Bretos, 1978).

TABLE I

Diameters of growth rings (mm) formed in Fissurella crassa *through the seasons.*

Initial size	Summer	Fall	Winter	Spring	Summer	Fall	Winter	Spring
34.0				35.5		43.5		48.5
34.1			43.9					
35.7	38.0		45.5					
35.8				46.5				
36.2			42.0					
36.7	41.4		46.0					
38.6	41.9		48.0					
38.7	41.5							
40.0			44.5		51.5			
40.4	42.0							
41.0		48.0						
42.0	45.8		51.5					
42.3			47.9		51.5			
43.6	47.2		50.0					
43.9	47.5		53.5					
44.6	46.5		53.4					
44.6	49.6							
44.7	49.0		59.0					
44.8	46.5		51.6		55.0		57.4	
45.3	47.5		55.0					
45.8			52.4		58.3			
45.8			53.8					
45.9			53.9		59.5		61.7	
46.3	49.5							
46.6	51.0		58.0					
46.7			51.4					
48.1			49.0		55.5			
48.2	52.6		60.0					
48.6	51.5							
49.3	51.4							
49.4	51.5							
50.0	57.5							
50.7	56.5							
51.1			53.3					
52.0			54.3					
52.8	56.0							
52.9	57.8							
53.1			57.2					
53.5			54.5		61.6			
55.6			60.0					
55.9			57.7					
55.9			63.8				69.0	
56.5	58.8							
58.7			68.0		66.4			
60.7	62.0		68.2					
62.6	64.6		68.8					
66.8	68.0		72.5					
70.0			71.8					
70.5	71.5		74.7					
75.0				76.6		78.0		

The concentric rings formed during shell growth were called growth rings. The formation of each new growth ring in tagged animals was registered (Table I). Shell lengths and ring diameters were measured to the nearest 0.1 mm using vernier calipers, on an imaginary antero-posterior axis passing through the apical hole. Initial shell lengths ranged from 34 to 75 mm.

The number of recaptured animals decreased with time. Data presented here concern 50 animals observed over 6–18 months, and which presented well defined growth rings on their shells.

Mean diameter and standard deviation of growth rings formed during the observation period are shown in Table II. Disturbance rings, assumed to be due to markings, were disregarded.

Growth and age were calculated following the methods described in Ricker (1975). Walford's graphic (Walford, 1946) was constructed using mean ring sizes to calculate maximum or asymtotic size. The least square regression of Walford plot has the general linear form: $Y = a + bX$. This expression becomes: $L_{t+1} = L_{\propto}(1 - k) + kL_t$. Here $X = L_t$, the size at time t; $b = k$, the slope of the Walford regression line; $a = L\ (1 - k)$, the Y-axis intercept from which L, the asymptotic size, can be calculated in the following derived expression: $L_{\propto} = (-L \propto (1 - k))/(k - 1)$. The von Bertalanffy curve for *Fissurella crassa* was fitted using the parameters obtained in the Walford regression. This curve is represented by the equation (von Bertalanffy, 1938):

$$L_t = L_{\propto}(1 - e^{-K(t+t_0)})$$

Sexual dimorphism in ring formation or growth in the experimental group could not be studied because sex could not be determined from external morphological features.

Control groups

Group A: A sample of 99 animals collected in the intertidal rocky shore of Los Lobitos (20° 28′ S) (Fig. 2) ranged from 19.8 to 59.8 mm in shell length, and 88% of them measured less than 47 mm. Shell lengths and ring sizes were

TABLE II

Growth ring diameters (and standard deviation) detected in Fissurella crassa *from Huayquique, Los Lobitos, and Los Verdes. Sizes underlined are assumed to correspond to annual growth rings.*

Growth rings	Los Lobitos			Huayquique			Los Verdes			Estimated age—years
	n	Mean diameter (mm)	s.d.	n	Mean diameter (mm)	s.d.	n	Mean diameter (mm)	s.d.	
I	—	—	—	—	—	—	—	—	—	—
II	65	26.0	± 2.7	—	—	—	—	—	—	1
III	28	35.1	± 1.9	5	35.8	± 1.5	47	36.3	± 2.0	1½
IV	8	43.3	± 1.7	17	44.5	± 2.5	104	43.5	± 2.0	2
V	5	51.4	± 2.0	30	51.2	± 2.4	109	50.9	± 2.5	2½
VI	1	56.6	—	20	58.1	± 1.8	112	57.8	± 2.1	3
VII	—	—	—	6	63.5	± 1.6	70	63.5	± 1.3	3½
VIII	—	—	—	6	68.5	± 1.3	87	68.4	± 1.3	4
IX	—	—	—	3	71.9	± 0.5	44	72.4	± 1.1	4½
X	—	—	—	2	75.7	± 1.3	28	75.4	± 0.9	5
XI	—	—	—	1	78.0	—	20	78.2	± 1.0	5½

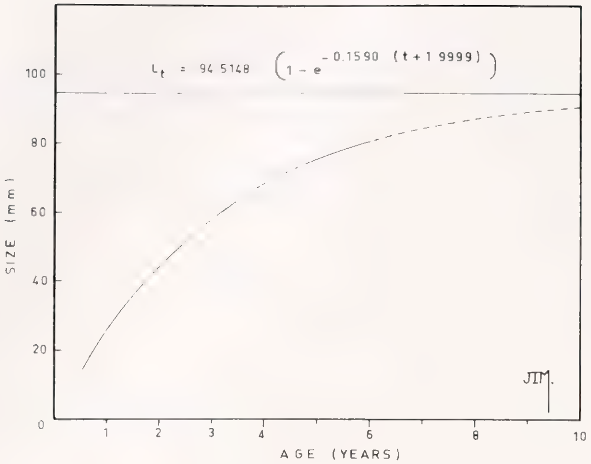


FIGURE 3. Growth curve of shell length for *Fissurella crassa* at Huayquique.

measured as in the experimental group. Mean ring sizes and standard deviation were calculated (Table II).

Group B: It was impossible to find large living specimens of *F. crassa* due to man's intensive predation on this species in northern Chile. For this reason, a second comparative group was analyzed for growth ring sizes in middle- and large-sized specimens. Fossil shells of *F. crassa* were collected in an archaeological site at Los Verdes (20° 26′ S) (Fig. 2). This sample of 189 shells ranged from 44.7 to 90.3 mm in shell length. Shell lengths and growth ring sizes were measured as indicated above. Mean ring sizes and standard deviation were calculated (Table II).

RESULTS

A disturbance ring formed on shells marked with notches. These rings were not considered in age and growth estimates.

Two periodic rings were formed per year in *Fissurella crassa* at Huayquique, except in one case where only one ring was formed during a year (asterisk in Table I). Ring formation occurred usually in winter and summer (45 cases), rarely in spring or/and autumn (4 cases) (Table I).

Formation of rings III–XI was observed during the study period in the experimental group at Huayquique. Sizes of these rings were used to determine age and growth. The Walford regression obtained using these data was: $L_{t+1} = 13.9007 + 0.8529\ L_t$, with correlation coefficient = 0.999 significant at $P = 0.005$.

Growth and age of *Fissurella crassa* calculated according to the von Bertalanffy equation are shown in Figure 3. Asymptotic shell length of this species at Huayquique was estimated at 94.5 mm.

According to these results, the animals in the experimental group were 1.5–5.5 years old (Table II).

Shells of animals of the group from Los Lobitos showed rings II and III, and occasionally rings IV, V, or VI (Table II). Rings III–XI were distinct on shells from Los Verdes (Table II). Other rings were also present in this group, but deciphering them was confusing. In middle- and large-sized specimens, the first ring is often obscure or disappears due to wearing away of the shell or to enlarging of the apical hole. Rings beyond the eleventh are not always clearly defined on account of slow growth at this age.

Mean ring sizes in the three groups were similar.

Discussion

Bretos (1978) experimentally determined the growth rate in *Fissurella crassa* in its natural habitat at Huayquique, Northern Chile. This species shows a shell length increment of 19.8 mm in the second year of life, 14.5 mm in the third, 9.7 mm in the fourth, and 6.8 mm during the fifth year (Fig. 3). These values are similar to the size differences (13.7 mm, 10.3 mm, and 7.2 mm) observed between odd rings formed in the experimental group in the present study (Table II).

According to the mean ring sizes obtained in the present investigation, a 1-year-old *F. crassa* has a shell length of about 26 mm (Table II). By adding the annual mean shell increment (Bretos, 1978) to the shell sizes at ages 1–5 (Table II), one obtains shell lengths very close to those calculated using the von Bertalanffy equation (Table III).

These results agree with those of Bretos (1978), demonstrating that *Fissurella crassa* exhibits a seasonal growth pattern, with diminishing growth rates during winter and early spring and in summer or autumn, bringing about formation of growth rings.

Other molluscs can form two rings per year on their shells, for example, the bivalves *Chlamys varia* (Dalmon, 1935), *Dreissena polymorpha* (Morton, 1969), *Corbicula fluminea* (Morton, 1977), and *Tellina fabula* (Salzwedel, 1979). Picken (1980) reports the formation of two types of bands on the shell of the patellid limpet *Nacella* (*Patinigera*) *concinna,* dark bands denoting winter shell growth and light bands, summer shell growth; so that 1 year's growth is represented by a pair of bands. In molluscs with two phases of shell growth, slowing of shell growth in winter can be attributed to a decrease in water temperature (a time when soft tissues can grow faster) (Salzwedel, 1979), whereas the reduced shell growth rate observed in summer could be related to metabolic changes or may coincide with spawning (Dalmon, 1935; Morton, 1969; Salzwedel, 1979).

No information is available on reproductive, physiological, or biochemical processes in the keyhole limpet *Fissurella crassa* to explain its observed growth pattern.

It can be concluded that *F. crassa* at Huayquique, northern Chile, has two growth phases a year, resulting in formation of two periodic growth rings, at

Table III

Comparative shell lengths according to annual growth increments (Bretos, 1978) and mean annual ring sizes. Growth ring numbers are in parenthesis.

Age (years)	Annual increment (mm)	Shell length (mm)	Annual ring size (mm)
1	(25.8)	(25.8)	25.8 (II)
2	19.8	45.6	44.5 (IV)
3	14.5	60.1	58.1 (VI)
4	9.7	69.8	68.5 (VIII)
5	6.8	76.6	75.7 (X)

least in animals from 1–6 years old. Unfortunately, the shell growth ring method of determining age could be applied only over a limited size range, because in older specimens shell growth is slow and rings are hard to identify. The largest specimen analyzed in this study had a shell length of 90.3 mm and a calculated age of about 10 years. Longevity of *F. crassa* in other regions of Chilean coast could be different since most of the *Fissurella* species living in Northern Chile attain greater sizes in central Chile.

ACKNOWLEDGMENTS

I am indebted to my husband, Mr. José Ignacio Moraga, for encouragement throughout the investigation and for making the figures. Thanks are also due to members of the Developmental Biology and Malacology Group for their help in the laboratory and in the field. This work was supported in part by SERPLAC I Región (Regional Development Funds).

LITERATURE CITED

BERTALANFFY, L. VON, 1938. A quantitative theory of organic growth (Inquiries on growth laws. II.) *Human Biol.,* **10**: 181–213.

BRETOS, M., 1978. Growth in the keyhole limpet *Fissurella crassa* Lamarck (Mollusca: Archaeogastropoda) in Northern Chile. *The Veliger,* **21**: 268–273.

BROUSSEAU, D. J., 1979. Analysis of growth rate in *Mya arenaria* using the von Bertalanffy equation. *Mar. Biol.,* **51**: 221–227.

COE, W. R., 1947. Nutrition, growth and sexuality in the pismo clam (*Tivela stultorum*). *J. Exp. Zool.,* **104**: 1–24.

DALMON, J., 1935. Note sur la biologie du pétoncle. *Rev. Trav. Off. Pêches marit.,* **8**: 268–281.

GORDON, J., AND M. R. CARRIKER, 1978. Growth lines in a bivalve mollusk: Subdaily pattern and dissolution of the shell. *Science,* **202**: 519–521.

JONES, D. S., I. THOMPSON, AND W. AMBROSE, 1978. Age and growth rate determinations for the Atlantic surf clam *Spisula solidissima* (Bivalvia: Mactracea), based on internal growth lines in shell cross-sections. *Mar. Biol.,* **47**: 63–70.

MORTON, B. S., 1969. Studies on the biology of *Dreissena polymorpha* Pall. 3. Population dynamics. *Proc. Malacol. Soc. Lond.,* **38**: 471–482.

MORTON, B. S., 1977. The population dynamics of *Corbicula fluminea* (Müller, 1974) (Bivalvia: Corbiculacea) in Plover Cove reservoir, Hong Kong. *J. Zool. Lond.,* **181**: 21–42.

NEWMAN, G. G., 1968. Growth of the South African abalone *Haliotis midae. Invest. Rep. Div. Sea Fish. S. Afr.,* **67**: 1–24.

PICKEN, G. B., 1980. The distribution, growth and reproduction of the Antarctic limpet *Nacella (Patinigera) concinna* (Strebel, 1908). *J. Exp. Mar. Biol. Ecol.,* **42**: 71–85.

RICKER, W. E., 1975. Computation and interpretation of biological statistics of fish populations. *Bull. Fish. Res. Bd. Can.,* **191**: 1–382.

SAKAI, S., 1960. The formation of the annual ring in the shell of the abalone *Haliotis discus* var. *hannai* Ino. *Tohoku J. Agric. Res.,* **11**: 239–244.

SALZWEDEL, H., 1979. Reproduction, growth, mortality, and variations in abundance and biomass of *Tellina fabula* (Bivalvia) in the German Bight in 1975/76. *Veröff. Inst. Meeresforsch. Bremerh.,* **18**: 111–202.

WALFORD, L. A., 1946. A new graphic method of describing the growth of animals. *Biol. Bull.,* **90**: 141–147.

WILBUR, K. M., AND G. OWEN, 1964. Growth. Pp. 211–242 in K. M. Wilbur and C. M. Younge, Eds., *Physiology of Mollusca.* Volume I. Academic Press, New York.

Reference: *Biol. Bull.*, **159**: 613–625. (December, 1980)

COURTING BEHAVIOR IN A SYNCHRONOUSLY FLASHING, AGGREGATIVE FIREFLY, *PTEROPTYX TENER*

JAMES F. CASE

Department of Biological Sciences, University of California, Santa Barbara, CA 93106

ABSTRACT

Display, courting, and mating behavior of the aggregative, synchronously flashing firefly *Pteroptyx tener* were analyzed with the aid of high-gain video recording. Males flash synchronously with other males under a variety of conditions, including flight and many phases of courtship, competition with other males, and mating. The courting male perches on the back of the female, rocks backwards and forwards, flashes his lantern directly into her eyes, and strikes her abdomen with his hind pair of legs. Characteristic flash exchanges occur before copulation, which is also accompanied by flashing by both sexes. The behavior of interloping males and the counter-behavior of the primary male are described. The import of these behaviors is discussed in relation to theories of the evolution and adaptive significance of synchronous flashing. It is suggested that the brilliant illumination of the female's eyes by the male may prevent her from seeing signals from other males or they from recognizing her emission.

INTRODUCTION

Synchronous flashing by large groups of male fireflies of several species of the Asian genus *Pteroptyx* is associated with aggregation of males and females together in trees and is believed to facilitate mating. The flashing has been studied visually and recorded from individuals and groups of fireflies in field and laboratory (Bassot and Polunin, 1967; Buck and Buck, 1968; Hanson, 1978; Hanson *et al.*, 1971; Lloyd, 1973b) and several speculations concerned with how flash synchronization might function during courtship and mating have been advanced (Buck and Buck, 1966, 1978; Lloyd, 1973a, 1979). However, aside from occasional sightings of antennation, mounting, or copulation, no observations have been made of actual courtship, here defined as interactions between male and female after physical contact and before intromission.

The dearth of crucial data about reproductive behavior in habitually synchronizing firefly species is easily understandable. In the first place the fireflies are minute (most species are 5–7 mm long, with light organ areas of only a few mm^2; Figs. 1a, b). Second, flash duration is usually very brief (40–100 msec). Third, the light, though of low absolute intensity, is dazzling when it impinges suddenly on the dark-adapted human eye at close range. Hence, even if the observer is not distracted by the simultaneous flashes of the many nearby fireflies in the swarm, the problem of focusing on a tight pair of small insects at a somewhat uncertain point in space during an instant of illumination in otherwise total darkness is considerable. To record sexual behavior instrumentally has also proven impossible, prior to the present investigation, because of inadequate spatial resolution. These

Received July 25, 1980; accepted September 25, 1980.

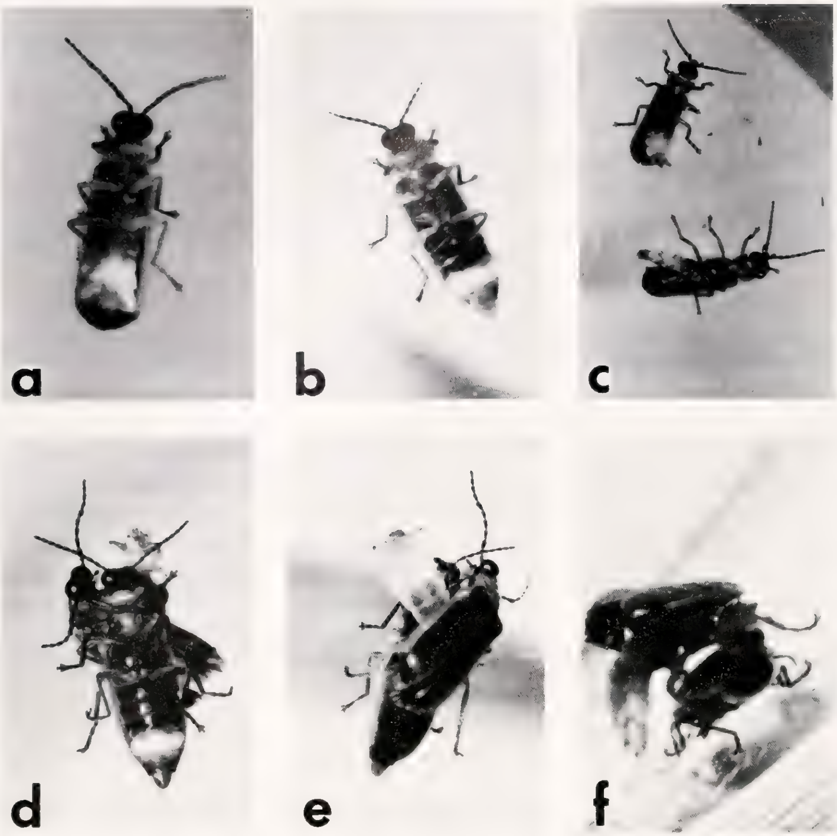


FIGURE 1. Individuals of *Pteroptyx tener* photographed in darkness with an electronic flash (×5). (a) Male (5.2 mm body length), ventral view, showing single luminous organ covering surface of sixth abdominal segment and two smaller, roughly triangular lateral organs in the seventh segment. (The mostly white fifth segment is not luminous.) (b) Female, ventral view showing luminous organ occupying sixth abdominal segment. (c) Two males, ventral view. Male at bottom of picture is displaying to the other male by twisting his abdomen so as to direct the light at the other. (d) Courting pair, ventral view. The male is standing on the back of the female in head-to-head alignment, and has flexed and twisted his abdomen almost 180° to bring his light organs close to, and almost directly over, the female's eyes. (e) Courting pair, dorsal view, illustrating how the male's abdomen (flexed to the readers left) is placed close to the female's eyes and showing also that neither of his hindmost pair of legs is used to hold on to the female. (f) Courting pair, rear end view. Male's body axis is turned about 15° to the left with respect to the female's and his abdomen is flexed forward (reader's left) and twisted to aim the light organ toward the female's head. Again the metathoracic legs are seen not to be used in grasping.

technical difficulties have now been overcome by the twin expedients of visual observation and video targeting via red light, to which fireflies are essentially blind, and of recording luminescence via a portable, high sensitivity, close focus, video camera. These techniques have revealed several close range male courting and competitive behaviors that are not only remarkably complex but appear to provide one or more plausible rationales for the function of flash synchronization in courtship and mating.

MATERIALS AND METHODS

Fireflies, *Pteroptyx tener* (Olivier) (Figs. 1a, b), were observed, and collected alive for darkroom study, from aggregations occurring along the lower (tidal) 10 km of the Kuala Selangor River, Selangor, Malaysia, in March, 1980. The fireflies were viewed from a boat and from a platform built in the dense riverside vegetation where large nightly displays of *P. tener* occur.

Light emission and other behavior was recorded with a Panasonic WV-1350A video camera (Matsushita Communications Industrial Co. Ltd.) equipped with a Panasonic "Newvicon" video tube having a light sensitivity of about 0.03 fc in the visible spectrum and a markedly greater, but unmeasured, sensitivity in the far red. Fitted with a 16 mm lens of f/1.6 aperture, this equipment recorded flashing behavior up to a distance of about 4 m. By moving the video tube relative to the lens, focus could be varied from about 5 cm to infinity. Illumination of the scene with a 1.5 V flashlight provided with a deep red filter (Wratten No. 89B, 670 nm cut-off) allowed excellent viewing of behavioral details with little or no disturbance of the insects, as might be expected from the observations of Lall *et al.*, 1980, who found that the sensitivity of the compound eye of the male of *Photinus pyralis* at 650 nm is only about 5% of its maximum value at 570 nm.

Video data and oral comments were recorded on a Panasonic VHS recorder and monitored with a Sony AVF-3250A electronic viewfinder. The entire system was powered by an AC converter and a 12 V car battery.

For viewing luminescence at farther than 4 m the video camera was coupled with an image intensifier (Varo, Inc.) providing a light gain of about 40,000.

It proved very difficult to document detailed and complete behavioral episodes in the field, owing to wind movement of vegetation and to the incessant movement of other fireflies near perched, displaying males. However, it was found that after fireflies had been in light for a few hours, exposure to darkness at any time of day triggered normal flashing activity. Consequently, detailed recordings were made in a darkroom and confirmed by visual observation and video recording of parts of behavioral sequences in the field. Fireflies used in the darkroom studies were netted just before we left the river each evening and transported in large plastic bags. Within a few hours males and females were separated and left overnight in light. The next day in the darkroom appropriate numbers of males and females were released into a terrarium supplied with fresh foliage. Synchronous flashing began within 0.5 hr of darkening the room; courting and mating soon followed. Darkroom temperature fluctuated around 21°C while evening temperatures on the river were in the range of 27°–28°C.

Still photographs of insects were made with a hand-held 35 mm camera and 105 mm macro lens by triggering a strobe while focusing on flashes in darkness. The strobe was never used near a firefly swarm under investigation and fireflies illuminated by the strobe were never used again.

Temporal relationships of flashing by courting and mating pairs were analysed by the following procedures. Appropriate video-taped flashing sequences were displayed on a video monitor at approximately 5× magnification. Hand-held 0.5-mm-diameter light guides (Edmund Scientific) were positioned to record images of the light organs of two fireflies. The light guides conducted light to a pair of photomultiplier tubes whose outputs led to a Grass polygraph through current to voltage converters. Adjustment of the video brightness and contrast allowed recording of remarkably clean signals. Time resolution was limited to 16 msec by the video frame refresh time. This was a small price to pay for the tremendously increased spatial resolution of the method, which made it possible to record separately the emissions of two light organs a few mm apart from a distance of 20 cm.

Both in 1979 and 1980 *Pteroptyx tener* males and females were also studied in a darkroom in Santa Barbara, where they lived and behaved normally for up to 6 weeks.

The observations described below are based on 4 hr of video recording in the field and in the Kuala Lumpur darkroom. Four complete matings were observed, three in the darkroom and one in the field. Many incomplete courtships, male to male display encounters, and interactions between interloper males and courting pairs were recorded in both field and darkroom.

RESULTS

General aspects of behavior

Synchronous flashing becomes established rapidly at dusk as males and females, with little flashing, fly directly out from river bank vegetation and assemble in trees along the water's edge, most frequently the mangrove *Sonneratia caseolaris*. The first flashes were seen at about 7:15 p.m. local time, when light on the open river under a cloudless sky was about 0.4 $\mu W/cm^2$ as measured with an upwards-directed photometer (United Detector Technology). These flashes were from males, usually perched at the tips of leaves. The frequency of flashing was about 3.7 flashes/sec (period 0.27 sec) at the typical sunset environmental temperature of 28°C. This is at or near the frequency of the full mass display by thousands of insects up and down the river, which is established within the next 0.5 hr and continues for much of the night. The synchronized flashing is produced wholly by males and is highly precise, typically being visible only in the first, 22nd, and 23rd frames of each successive 23 video frames.

The synchronized males in the trees engage in two types of flashing, differing in flash intensity and duration, and in body attitude while flashing, but not in frequency. Much time is spent sitting or walking slowly, making rhythmic single flashes with the abdomen held parallel to the leaf surface. The emission in these flashes is relatively dim, may be as short as 70 msec, and may involve only the most anterior parts of the lantern (Fig. 1a) or in any event be initiated in the anterior organ. At irregular intervals the male changes to display flashing, which occurs usually with the male standing prominently on the edge or tip of the leaf, and in which the abdomen is twisted longitudinally and usually somewhat flexed so that his light is directed not down toward the leaf surface but laterally. During display flashing the intensity of light is much greater than in non-display flashing. The flashes may be up to 300 msec in duration and be multiple-peaked, so that the result is a virtually continuous, pulsating light emission (Fig. 2). Video records show that the multipeaked display flash is initiated by a low intensity flash from the most posterior parts of the lantern followed by a brilliant flicker of the whole organ.

Displaying to nearby males is a major male activity. During such display the light organ is aimed so that its surface is roughly perpendicular to a line from the displaying male to the other. The aiming is usually lateral (Fig. 1c) but a different direction was seen in a video sequence showing a male displaying while running to a pair in courtship. In this instance the approaching male directed his light forward

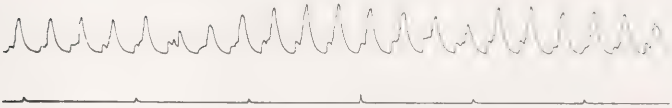


FIGURE 2. Photomultiplier record from video record of a male displaying in the field. Each compound flash begins as the the preceeding one dies away. Average flash duration = 0.28 sec; period = 0.28 sec; time marks = 1 sec.

towards the pair while the male of the pair displayed towards him. The trigger for male-male display can be purely photic, as a captive male will display at his own reflection from a mirror.

When not responding to a nearby male, a displaying male aims his light away from the mass of synchronizing fireflies. Individual males do not display for more than a few minutes at a time but whether displaying or not displaying, and sometimes even when flying (Fig. 3), they maintain flash synchrony with the other males of the swarm. The only exceptions are rapid flickers made when they are disturbed, and certain brief episodes of flashing during courtship (see below).

Females never flash in synchrony with males. They produce single-peaked flashes lasting up to nearly 1 sec, usually at a much slower rate than the male's display frequency and nearly always in an irregular pattern (Fig. 4). Such behavior is markedly obvious in the mass of displaying insects. Females may emit continuous glows when mechanically disturbed. Although the female light organ spans the width of the abdomen (Fig. 1b) the entire organ is virtually never active. Usually only the outer edges flash, giving the impression of a pair of luminous dots (Fig. 7g).

Further details of the biology of *P. tener* will be presented elsewhere.

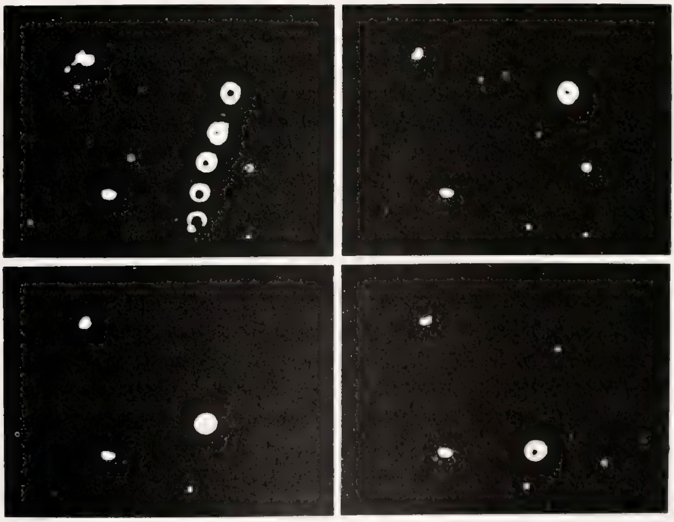


FIGURE 3. Flash synchrony in flight. Frame at upper left is a composite video record showing five successive flash positions (largest images) as closer male flew from top to bottom of field, flashing in synchrony with several perched males. The other three photographs are of single video frames from the sequence, showing that the flying male flashed each time in coincidence with the perched individuals. None of the video frames separating the frames showing flashes (there being about 22 frames between episodes) showed any flashing.

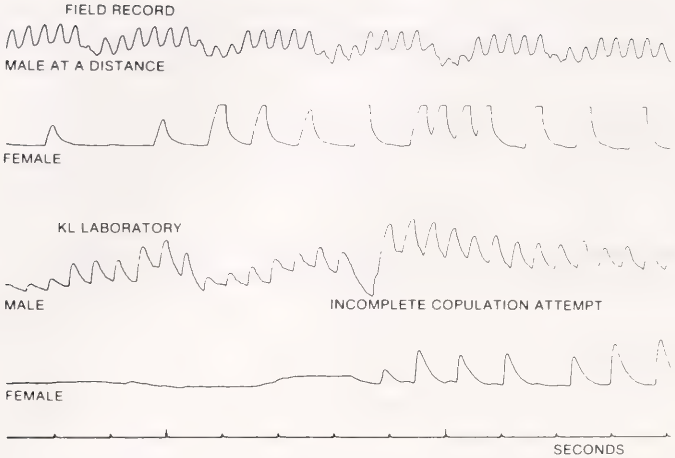


FIGURE 4. Luminescence of female in relation to different male behaviors. Top two traces show flashes of a non-displaying male and concurrent flashes of a female 10 cm distant from him. Bottom two traces show display flashes of mounted male, including those during a copulation attempt, and the concurrent luminescence of the female. Flat flash peaks in second trace are artifacts and the slow baseline shifts in all records are caused by variation in video background illumination.

Courtship and mating

Courtship begins when the male, flashing synchronously with the swarm, or not flashing at all, rapidly approaches a female, who may or may not be flashing. Video records show that walking males tend to make their first contact with females by touching their heads to the end of the female's abdomen. He climbs upon her back with his head facing in the same direction as hers and maintains his perch with only his first two pairs of legs. His abdomen is acutely flexed and twisted so that his light organ is held just above the head of the female (Figs. 1d, e). He continues or begins flashing, along with frequent bouts of waving his light organ over the female's head, while simultaneously striking her abdomen with his last pair of legs. The male's abdomen is held a short distance from the head of the female (Fig. 1f) but never seems to touch her until copulation is attempted. These acts are accompanied by rapid fore and aft swaying of the male's entire body parallel to the female's body axis. In the darkroom, courtship sometimes lasted as long as half an hour before ending in copulation or in separation of the pair.

During all this male activity the female may stand quietly or may walk about. She may luminesce or not. When luminescing she sometimes increases her flash frequency even above that of the male, and at times her flashes may roughly alternate with the male's emissions (Fig. 5). In the field this joint luminescence is often what first brings to notice the presence of a courting pair. The female seems to make few other visible responses to attentions from the male, and within 30 sec after his departure from an aborted courtship her flashing has slowed to well below the typical male frequency. In one video sequence the female used a leg to rub her eye and then to push at the male's brilliantly flashing light organ. Occasionally the abdominal striking seemed to cause the female to lift her abdomen. This did not seem to be strongly correlated with attempted copulation, however.

Courtship is interspersed with copulation attempts. Initiation of an undisturbed copulation attempt is signalled by 2 or 3 sec of darkness followed by a short burst

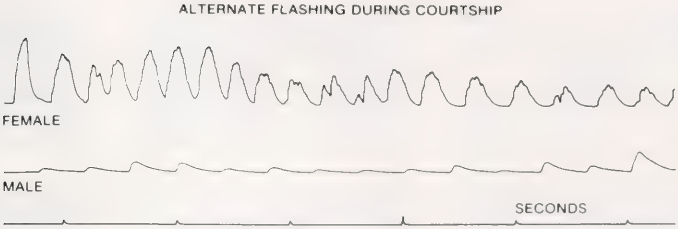


FIGURE 5. Flashing of a courting pair, showing occasional flash coincidence and more frequent alternate flashing of male and female.

of especially brilliant and more slowly delivered flashes by the male, whose lantern still is held over the head of the female (Fig. 6, third trace). The female commonly begins to flash, if she is not already doing so, just before the male's brief dark period (Fig. 6, fourth trace). The male, continuing to flash intensely, then rapidly moves his abdomen to cover the female's terminalia from above and attempts to copulate (Fig. 7). Unsuccessful copulation attempts last only a few seconds before the male instantly returns to brilliant flashing and vigorous striking at the female's abdomen for a brief interval before trying again.

No consistent cue for an undisturbed male to switch from flashing at the female's head to attempting to copulate was detected. The one definite trigger to an attempt is approach of another male, which invariably causes the courting male to increase the intensity of his flashes (Fig. 8) and to rapidly alternate the abdomen between the head flashing posture and the copulation initiation position, in which the male terminalia cover the female's from the dorsal aspect.

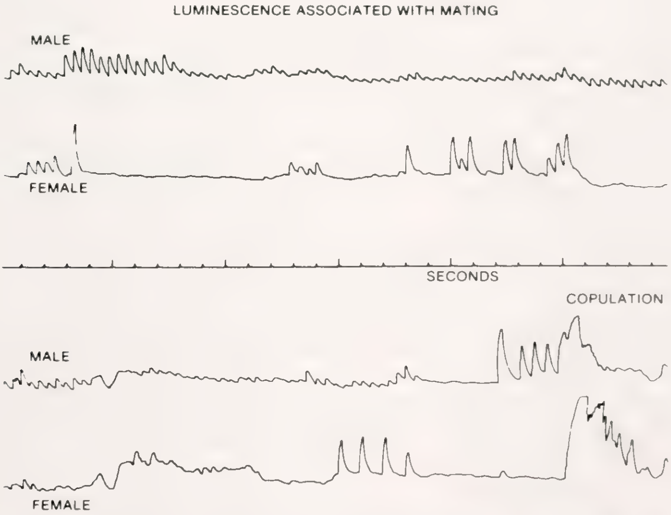


FIGURE 6. Luminescence during 50 sec preceding mating. Upper pair of records continuous with lower pair. Male maintained continuous low intensity flashing after an initial episode of bright flashes accompanied by abdominal striking (visible on video). Just before copulation his flashing ceased for about 3 sec immediately before four maximal flashes occurring simultaneously with his abdominal movements to effect copulation. Subsequent to these flashes both male and female flashed brightly but their contributions could not be resolved separately by the light guides. The four bright flashes by the female just before copulation were unique to this mating. From video recording in Kuala Lumpur laboratory.

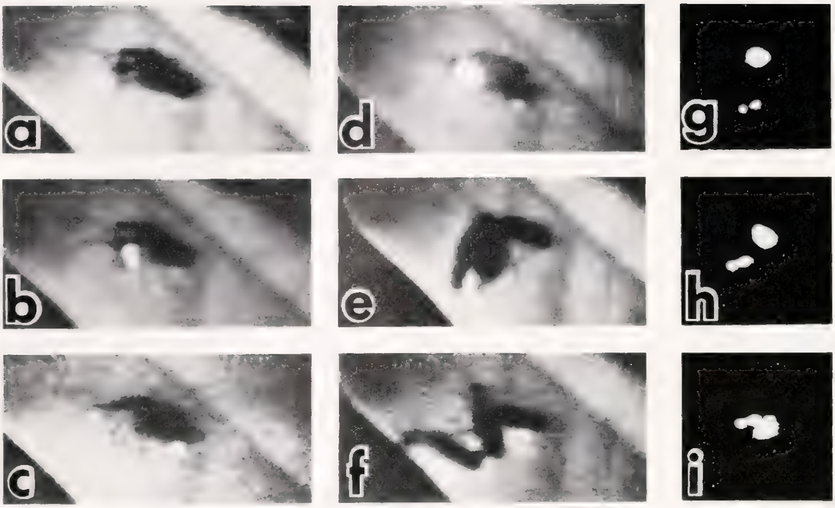


FIGURE 7. (a–f) Single video frames of the copulation recorded in Figure 6. (a) male perched on female with lantern held over her head, neither flashing; (b) male flashes, with his light appearing paired since it is seen through and around his abdomen; (c) female flashes; (d) both flash; (e) the instant of copulation with both members of pair flashing as interloper arrives from right; (f) pair rotates still flashing, male at lower left, female center, interloper at right, flashing and about to initiate courtship. (g–i) Three video frames of another copulation viewed through glass from below; (g) courtship position, male's lantern at top; (h) male moves tail towards female genitalia to start copulation; (i) copulation in progress.

Once copulation is accomplished, both male and female continue to flash brilliantly, often in alternation. Within a minute the pair rotates to a tail-to-tail position, remaining in copulation for at least several hours. Flashing rapidly subsides and the pair commonly takes up a more sheltered position. They are then difficult to induce to flash, even by an interloping male (Fig. 9).

How the male distinguishes between male and female when initiating courtship is not clear. The discriminatory powers of the normal male may not be especially acute, as the field video records show one instance in which a male courted and attempted copulation with the male of a courting pair before disengaging and exiting, still flashing. Similarly, another video record, made in the darkroom on the day after collecting, shows a successful copulation occurring simultaneously with arrival of an interloper who undertook active courtship with the female immediately after the copulators rotated (Fig. 7). This interloper remained with the pair for at least 3 hr.

Under laboratory conditions in Santa Barbara, courtships between males were common even when many females were present. The ridden member of such male-

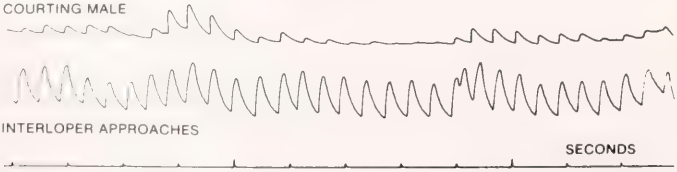


FIGURE 8. Flashing of courting male before (top trace) and after (bottom trace) interloper approaches, showing brightening of flashes.

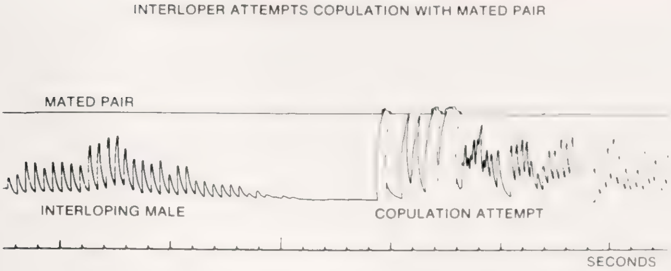


FIGURE 9. Second (interloper) male attempts copulation with rotated mating pair. Neither member of mating pair emits any light (bumps in trace are artifacts caused by pen from lower trace). Note that this attempt to copulate with a female *in copulo* has the characteristic form of a normal mating attempt.

to-male pairings continued to behave normally, even making typical male displays to passing males. This behavior was not observed in the field, and so may not be normal. While normal synchronizing behavior and low mortality characterized groups of insects maintained in Santa Barbara for up to 45 days, no complete copulations were seen among those insects.

Events subsequent to mating remain obscure. Pairs remain *in copulo* for hours, perhaps all night. Both males and females leave the display sites in increasing numbers towards dawn and fly into nearby vegetation just above the high tide mark. Early in the evening females are seen in small numbers along the river bank near the display sites. They are invariably flying slowly and hovering a few inches off the ground while making long, single-peaked flashes. They are perhaps getting ready to oviposit. Males are never seen during the night in such places.

DISCUSSION

The present study, in showing that *Pteroptyx tener* males engage in at least three modes of in-tree flashing, in addition to at least three close range interactions with females, has enlarged the male's known repertory while at the same time showing how much remains to be discovered. The male display flashes aimed outward from the tree are very probably long-range recruiting or convocational signals, as assumed in other synchronizing species, but whether they attract males and females indiscriminately or specifically remains unknown. No instance of a female coming to a male, or indeed engaging in any type of long range or mid-range signaling with a male was recognized. Neither was any clue found as to the significance of the dim, un-aimed flashing which occupies a major part of the male's flashing time. The bright display flashing directed at nearby males, however, is clearly suggestive of a competitive or comparison display that often ends with what the observer takes to be defeat and departure of one individual. It is thus quite similar to the aggressive flashing between pairs of walking *P. malaccae* males reported by Buck and Buck (1978), but without the actual physical combat seen in that species. In neither *Pteroptyx*, however, is there any actual evidence that females select males on the basis of such a competition. Rather, its visible consequence would seem to be the regular spacing of displaying males through the vegetation.

Competition among males displaying to a female on the basis of comparative flash intensity, suggested for *P. malaccae* by Buck and Buck (1978), seems excluded in *P. tener*, since displaying males were not grouped in relation to a female. No behavior suggesting one male preempting another's place in a dialogue (Lloyd's 1973a "interloping") was seen. In fact, no male-female interaction prior to actual contact was detected in *P. tener* except the possibility that pheromonal attraction draws the male to the end of the female's abdomen just prior to mounting, in the sort of "change in communicative channel" postulated by Lloyd (1973a). However, none of the other possible pre-mounting female behaviors or sexual interactions considered by Lloyd seems to occur. The female of *P. tener* seems singularly inactive once within the tree, in striking contrast with the female's precise photic role in dialogue in many roving fireflies, her active flight in others, and her participation in complex aerial sexual chases in still other species (*e.g., Luciola obsoleta;* Lloyd, 1972).

It is in close range male-female interactions that the present work has yielded the most new information. Courtship in *P. tener* is the most complex yet described and contrasts strongly with behavior in many non-synchronizing species in which the male, after locating the female by photic signaling, may copulate seemingly without further preliminaries and with little or no luminescence. Though there is no evidence of whether synchronizing species derive from non-synchronizers or vice versa, certain aspects of *P. tener* behavior may possibly have analogs among non-synchronizers. Lloyd (1964), for example, observed that the mounted male of *Pyractomena dispersa* "tapped the abdominal tergites of an apparently unresponsive female with his parameres. . ." before attempting copulation. If such tactile stimulation is a general releaser of receptivity the *P. tener* male's use of his hind legs for this purpose, rather than his genitalia, could be a compromise forced by the overriding need to hold his abdomen near the female's head. Another parallel may be the long periods of luminescent interactions between male and female in the New Guinea species *Luciola obsoleta*. Here Lloyd (1972) noted a series of very bright flashes from the male before or while mounting. Although copulation was not seen to occur, mated pairs were found in the tail-to-tail position with both members glowing or flashing. *L. obsoleta* might be considered intermediate in courting behavior between roving fireflies and the aggregative synchronizer *P. tener*. The genus *Luciola* contains at least one synchronizer, *L. pupilla*, and *L. obsoleta* is somewhat aggregative, though not synchronic.

The coupling of abdominal movement with flashing is widespread in the Lampyridae, and lantern aiming by the displaying *P. tener* male may have its roots in the almost universal practice of the females of dialogue species of aiming their lanterns towards the male while answering. Further, Case and Buck (unpublished) have found that the male of *Photinus greeni* points his lantern away from the substrate while flashing prior to taking flight. However, the holding of the male's lantern over the female's head while flashing is a major element of *P. tener* courtship that seems totally without parallel in firefly behavior and invites inquiry into its possible physiological consequences.

First, flooding the female's head with light may reduce her dark adaptation and thereby limit her ability to see luminescences of other males. To be sure, the mounted male continues to synchronize with other males, showing that he is not self-blinded, but in *Pteroptyx cribellata* it is known that the time of the male's flashing is the time of his minimum sensitivity to visual input (Buck *et al.*, unpublished). In any case, if the male's eyes are twice as far from his lantern as the female's (Figs. 1e, f) his chances of being dazzled would be only one quarter of hers. Second, point-blank discharge, by the mounted male, of long-duration display flashes directly into the female's eyes is likely to be perceived as a sinusoidally varying illumination that would mask dimmer, more discrete flashes of other, more distant, males that might possibly have communicative significance. A third possibility is that the male's flashing might, through recurrent pacemaker resetting (Hanson *et al.*, 1971; Hanson, 1978) or photic inhibition (Case and Buck, 1963; Case and Trinkle, 1968), alter any possibly attractive flashing by the female to a type less attractive or less obvious to searching males. Thus present records show that under courtship conditions female flashing may speed up and blur together with the male's flashes (Fig. 5). While male and female emissions are then still separable spatially by the human eye, or instrumentally (Fig. 9), a firefly might see this joint luminescence as a single strong male display. Hence the observation of a displaying male running up to a pair engaged in alternated flashing might have had territorial rather than sexual significance. Fourth, strong photic stimulation might be a necessary releaser for female copulatory behavior. This is suggested by the brief set of unusually brilliant and long flashes which immediately precede virtually every copulation attempt (Figs. 6, 9).

Though the long period of courtship, and occasionally observed unsuccessful terminations, suggest that testing is going on during the mounted phase of courtship it is not obvious how sexual selection is exercised by the female. Her flashing is erratic but there is a clear tendency for her to increase flash intensity and frequency, often associated with a crescendo of mechanical and photic stimulation by her partner. The only movement, aside from slow walking, that she seems to make is occasionally raising the end of her abdomen. The observation that the female often begins to flash immediately before the brief interruption in the male's flashing that just precedes his attempt to copulate suggests a receptivity cue, but this female behavior is not invariant nor are subsequent male attempts always successful. Furthermore, the increased frequency and brightness of her flashes might just as well be signals to attract other suitors, since her luminescence also increases in the presence of an interloper.

Any of the first three suggested effects of head illumination by the mounted male (above) appears to obviate photic selection of males by females in that phase of courtship. Yet as already mentioned, there is no evidence of signaling or selection before the male comes to close range, and it is hard to understand why the female often seems to remain passive when a male, without flashing, arrives and initiates courtship. The fact that the male is constrained to supply such an overwhelming photic stimulus to the female might mean that the ultimate stage of mate selection in *Pteroptyx tener* has shifted away from one in which the female's evaluation of male photic performance was paramount. Perhaps as a result of increasing population density, such as occurs in Malaysian *Pteroptyx* swarms, the female would tend to be in a continual state of photic confusion from the barrage of male display signals and thus sexual selection on the grounds of inspection of the luminescences of the male from a distance would break down. This seems a possibility because at least the female of *Photinus greeni*, while engaged in timing photic signals, integrates all those seen without making any spatial discrimination (Case and Buck, 1979). Consequently, the female's photic release threshold might rise markedly, necessitating point-blank stimulation, or the female might shift to non-photic selection criteria which the male must meet in addition to photically masking the female from the light of other males or disguising her flashes.

It is hoped that the discovery of the wholly unexpected and unpredicted apparent photic coercion of the *P. tener* female by the mounted male will stimulate new studies and re-evaluation of observations on other species. Particularly necessary are further efforts to understand the adaptive significance of the synchronized flashing of male fireflies. In *P. tener* and certain other species synchrony is all-pervasive in short range, long range, display, and non-display flashing, indicating that it has been genetically selected in a variety of interlocking contexts. However, known differences in the behaviors of different habitual synchronizers (Buck and Buck, 1978) and in their physiological control mechanisms (Hanson, 1978) caution against assuming that all synchronizing species have the same photic reproductive adaptations and behaviors.

This investigation has not touched on the cause of aggregation, but since the rhythm of flashing in habitually synchronizing species is controlled by a specialized pacemaker which becomes obligatorily entrained to rhythmic external photic signals (Hanson *et al.*, 1971), mass synchronous flashing depends on mass congregation, and the functional significance of males and females coming together in huge numbers is an integral part of the puzzle of synchronous flashing fireflies.

ACKNOWLEDGMENTS

I am most particularly indebted to John and Elisabeth Buck and to Frank E. Hanson for much help and advice. I gratefully acknowledge my introduction to *P. tener* by Dr. Ivan Polunin. In Malaysia I have been ably assisted over the past several years by Thian Heng Lee and, in 1980, by Devinder Kumar. Profs. J. Furtado, Y. C. Siew, and others of the Department of Zoology, University of Malaysia, have been most helpful. Jallaludin, of Kampong Kuantan, Malaysia, organized construction of the observation platform and provided transport on the river. Research support was provided by NSF Grant BNS76-80246, University of California Faculty Research Funds, the FBN Fund, and, in its early stages, by the Office of Naval Research.

LITERATURE CITED

BASSOT, J. M., AND I. V. POLUNIN, 1967. Synchronously flashing fireflies in the Malay Peninsula. *Sci. Rep. Yokosuka City Mus.*, **13**: 18–22.

BUCK, J., AND E. BUCK, 1966. Biology of synchronous flashing of fireflies. *Nature*, **211**: 562–564.

BUCK, J., AND E. BUCK, 1968. Mechanism of rhythmic synchronous flashing of fireflies. *Science*, **159**: 1319–1327.

BUCK, J., AND E. BUCK, 1978. Toward a functional interpretation of synchronous flashing by fireflies. *Am. Nat.*, **112**: 471–492.

CASE, J. F., AND J. BUCK, 1963. Control of flashing in fireflies. II. Role of central nervous system. *Biol. Bull.*, **125**: 234–250.

CASE, J. F., AND J. B. BUCK, 1979. Limitations on firefly mate selection by luminescent signalling. *Western Soc. Naturalists, Abstr, 60th Ann. Meeting*, 28.

CASE, J. F., AND M. S. TRINKLE, 1968. Light-inhibition of flashing in the firefly *Photuris missouriensis*. *Biol. Bull.*, **135**: 476–485.

HANSON, F. E., 1978. Comparative studies of firefly pacemakers. *Fed. Proc.*, **37**: 2158–2163.

HANSON, F. E., J. F. CASE, E. BUCK, AND J. BUCK, 1971. Synchrony and flash entrainment in a New Guinea firefly. *Science*, **174**: 161–164.

LALL, A. B., R. M. CHAPMAN, C. O. TROUTH, AND J. A. HOLLOWAY, 1980. Spectral mechanisms of the compound eye in the firefly *Photinus pyralis* (Coleoptera: Lampyridae). *J. Comp. Physiol.*, **135**: 21–27.

LLOYD, J. E., 1964. Notes on flash communication in the firefly *Pyractomena dispersa* (Coleoptera: Lampyridae). *Ann. Entomol. Soc. Am.*, **57**: 260–261.

LLOYD, J. E., 1972. Mating behavior of a New Guinea *Luciola* firefly; a new communicative protocol (Coleoptera: Lampyridae). *Coleopt. Bull.*, **26**: 155–164.

LLOYD, J. E., 1973a. Model for the mating protocol of synchronously flashing fireflies. *Nature*, **245**: 268–270.

LLOYD, J. E., 1973b. Fireflies of Melanesia: bioluminescence, mating behavior, and synchronous flashing (Coleoptera: Lampyridae). *Environ. Entomol.* **2**: 991–1002.

LLOYD, J. E., 1979. Sexual selection in luminescent beetles. Pp. 293–341 in Blum, M. S., and N. A. Blum, Eds. *Sexual selection and reproductive competition in insects.* Academic Press, N. Y.

Reference: *Biol. Bull.*, **159**: 626–638. (December, 1980)

THE INTRACELLULAR MECHANISM OF SALINITY TOLERANCE IN POLYCHAETES: VOLUME REGULATION BY ISOLATED *GLYCERA DIBRANCHIATA* RED COELOMOCYTES[1]

CHARLES J. COSTA, SIDNEY K. PIERCE, AND M. KIM WARREN

Department of Zoology, University of Maryland, College Park, Maryland 20742

Abstract

Glycera dibranchiata is an osmoconforming polychaete that lives in seawater osmotic concentrations ranging from 1366 to 374 mosm/kg. *G. dibranchiata* uses free amino acids (FAA) to reduce intracellular solute concentrations during hypoosmotic stress: Both body wall tissue and isolated red coelomocytes taken from worms adapted to dilute seawater for 14 days showed substantial decreases in several amino acids when compared to full-strength seawater controls. Measurements of cell volume made on isolated red coelomocyte suspensions during acute exposure to dilute media indicate that volume regulation is rapid but incomplete. The volume of red coelomocytes does not return to control values during 120 min. The volume recovery of the isolated coelomocytes during hypoosmotic stress is accompanied by salinity-dependent efflux of FAA proline and taurine. A comparison of this efflux with the FAA pool in full-strength-seawater acclimated cells indicates that the efflux is accomplished by a selective change in membrane permeability to proline and taurine.

Introduction

Euryhaline marine invertebrates which survive large changes in extracellular osmotic pressure have provided excellent models for studying the mechanisms underlying cellular volume regulation (Gilles and Schoffeniels, 1969; Lang and Gainer, 1969a, b; Gerard and Gilles, 1972; Pierce and Greenberg, 1972, 1973; Watts and Pierce, 1978; Amende and Pierce, 1980). Most of these studies used isolated solid tissues as experimental preparations. While the solid tissue preparations have yielded a great deal of information, they have the disadvantages that cell volume cannot be directly measured, and that access of the medium to the cells may vary with tissue thickness. A homogeneous suspension of large numbers of single cells (coelomocytes or blood cells for example), would solve both the volume-measurement and medium-access problems. However, suitable cells are relatively rare in invertebrates with sufficient salinity tolerance to be of use in cell-volume regulation experiments.

One such homogeneous cell suspension can be prepared from the coelomocytes of the glycerid polychaete *Glycera dibranchiata*. This worm is also very much a euryhaline osmoconformer, tolerating salinities from 12.4 to 46.5‰ (Machin,

---

Received July 8, 1980; accepted September 7, 1980.

Abbreviations used: FAA, free amino acid; ASW, artificial seawater; mosm, milliosmoles per kilogram water; MCV, mean cell volume.

[1] Contribution No. 157 from the Tallahassee, Sopchoppy, & Gulf Coast Marine Biological Association, Inc. Supported by the NIH Grant GM-23731.

1975). The coelomic fluid of *G. dibranchiata* contains three readily identifiable cell types. Most numerous, except in gravid individuals, is the nucleated, hemoglobin-containing coelomocyte (hereafter, red coelomocyte). These cells have been described both morphologically (Seamonds and Shumacher, 1972) and physiologically as oxygen carriers (Hoffmann and Mangum, 1970). Second, gametes, either eggs or sperm, are often present in numbers dependent on the breeding condition of the worm. Finally, always present, though in relatively small numbers, are non-pigmented amoebocytes. Machin and O'Donnell (1977) provided a preliminary description of the volume regulatory capacity of *Glycera* coelomocytes and found these cells able to partially recover cell volume following osmotic swelling during exposure to hypoosmotic media. The mechanism effecting the volume decrease was not examined.

As a first approach to identify the volume control mechanism of the red coelomocytes, we have characterized the water balance of both the animal and the isolated cell. We have investigated the effect of acclimation to hypoosmotic seawater on the FAA pool of *G. dibranchiata* body-wall muscle and of isolated red coelomocytes. We have also examined the volume regulatory responses of isolated red coelomocytes to both acute and long term hypoosmotic stresses. Finally, we have begun to identify the volume recovery mechanism used by *G. dibranchiata* coelomocytes. The results show that the FAA pool of both body wall muscle and red coelomocytes in acclimated worms declines in a salinity-dependent manner. Further, the response of the red coelomocyte to acute hypoosmotic stress is a transitory and rapid swelling followed by a substantial volume recovery accompanied by an efflux of the intracellular amino acids taurine and proline.

MATERIALS AND METHODS

Animals and solutions

Specimens of *Glycera dibranchiata* were purchased from the Maine Bait Co., Newcastle, Maine. The worms were shipped to College Park *via* air mail special delivery and routinely arrived in excellent condition. Upon arrival, single worms were placed into individual lengths of glass tubing, both ends of which were capped with plastic screen cloth. The screen allowed free flow of water through the tube, while preventing the worms from escaping. The worms in their tubes were placed into full-strength artificial seawater (ASW) (Instant Ocean, salinity = 35‰ = 1001 mosm/Kg H_2O; mosm hereafter) at 15°C. All experimental acclimation salinities were made by dilution of ASW with distilled water. The osmotic concentration of all solutions was determined before use with a freezing-point depression osmometer (Osmette, Precision Systems).

Coelomic fluid osmotic concentration

Worms were kept in ASW for 48 hr. Then a large group was transferred into 697 mosm ASW and allowed to acclimate to that solution for 48 hr. Care was taken to completely empty each tube and to refill each with the lower salinity water. Next, a portion of this group was similarly moved to 463, 374, and finally to 230 mosm ASW, all at 48-hr intervals. All worms in this last dilution died after 48 hr so no further dilutions were attempted, and the use of 230 mosm ASW as an experimental medium was abandoned. The worms were allowed to acclimate to the other salinities for at least 14 days, during which time no mortality occurred.

At the end of the 2-week acclimation period, five animals were removed from each test salinity together with a seawater sample, and the osmotic concentrations of the coelomic fluid from each worm and of the seawater were determined. The coelomic fluid was prepared as follows. The worm was removed from the glass tube, placed on a piece of filter paper and gently blotted to remove external fluid. The worm was then held over a beaker, and the body wall was cut in several places with scissors. Coelomic fluid was allowed to drain into the beaker, and the carcass was saved for subsequent determination of water and amino acid content of body wall tissue (see below). The coelomic fluid was then centrifuged at 5000 × g (4°C) to remove coelomocytes and debris. Occasionally, at this point, an individual sample would show evidence of coelomocyte lysis; such samples were discarded and replaced. The supernatant was then removed, and the osmotic concentration of that solution determined with the osmometer. Care was taken throughout the procedure to keep samples covered and cold to minimize evaporation.

The data were analyzed by standard regression techniques, resulting in the least-squares regression line and the 99% confidence belt for the regression line between 374 and 1001 mosm (Steel and Torrie, 1960). The slope of the regression line was compared to the slope of the isosmotic line (1.0) using Student's t test for slopes (Snedecor and Cochran, 1967).

Water and intracellular free amino acids in body wall tissue

Portions of the body wall were dissected from the carcass remaining from the above experiment and weighed on an analytical balance. The pieces of body wall were then frozen on dry ice, lyophilized overnight, and re-weighed. Tissue water as a percentage of the wet weight was calculated from the following expression: Tissue water = (wet wt − dry wt) · wet wt^{-1} · 100.

The dry tissue was homogenized in 40% ethanol in a glass homogenizer. The homogenate was brought to a boil to precipitate protein and then centrifuged at 20,000 × g for 20 min. The supernatant was lyophilized, the residue dissolved in an appropriate amount of lithium citrate buffer (pH 2.2) and the amino acid content of this last solution determined with an amino acid analyzer (JEOL, model JLC-6AH). The effect of acclimation salinity on the body wall intracellular FAA pool was tested by analysis of variance and the significant treatment means identified by the Student-Newman-Keuls multiple range test.

Free amino acid content of red coelomocytes

Another group of blood worms was acclimated to 1001, 695, 463, and 374 mosm ASW in the manner described above. After the 2-week acclimation period the coelomic fluid was collected from five worms in each test salinity, as already described. Hemoglobin-containing coelomocytes were isolated from other coelomocytes by centrifugation at 5000 × g (4°C) for 10 min. The red coelomocytes, now free of gametes and amoebocytes, were then resuspended in 2 ml of ASW at the osmotic pressure of acclimation. The cells were recentrifuged (1000 × g, 5 min, 4°C), washed three times and finally resuspended in 0.5 ml of ASW at the salinity of acclimation. At this point considerable lysis of the coelomocytes taken from worms acclimated to the lowest salinity (374 mosm) had occurred.

Thus, we were unable to measure accurately the amino acid concentrations of those cells. Lysis did not occur in coelomocytes from any other salinity. The hematocrit of this final suspension was usually 5–10%. Cell number in a 0.1-ml sample of each suspension was determined with an electronic particle counter (Electrozone Celloscope, Particle Data, Inc.). The remaining 0.4 ml of each suspension was recentrifuged (5000 × g, 10 min, 4°C) and the supernatant solution replaced with 1 ml of distilled water followed by 1 ml of 80% ethanol. This solution was brought to a boil and then centrifuged at 20,000 × g (4°C) for 20 min. The supernatant solution was lyophilized and the residue dissolved in an appropriate volume of lithium citrate buffer (pH 2.2). The amino acid content of this last solution was determined with the amino acid analyzer and concentrations expressed as nmol/10^6 cells. We have only reported concentrations of the acidic and neutral amino acids for body wall and coelomocytes, since preliminary analyses indicated that the basic amino acids, excepting arginine, were present in very low concentrations (<0.5 μmol/gm dry weight or 0.5 nmol/10^6 cells). Arginine concentrations were higher, but they did not vary systematically with salinity. Data from this experiment were statistically analyzed as in the previous section.

Identification of hypotaurine

Our preliminary amino acid analyses of both body wall and coelomocyte extracts included a substantial peak which exactly coeluted with the urea peak of our standards. Urea has a low color index with ninhydrin and, if the peak was in fact urea, its contribution to the total osmotically active solute pool in *Glycera* would be enormous. Amende and Pierce (1978) showed that a similar peak on chromatograms of extracts of the bivalve *Noetia ponderosa* was not urea but hypotaurine. Therefore, we performed experiments designed to identify this substance in the *G. dibranchiata* extracts. Using identical methods to those described by Amende and Pierce (1978), we determined that the *Glycera* "urea" peak was also hypotaurine.

Red coelomocyte volume regulation: the acute response

Red coelomocytes, collected from *G. dibranchiata* acclimated to 1000 mosm ASW, were isolated from contaminating cell types as described above. Following isolation, the red coelomocytes were resuspended in 996 mosm ASW (as formulated by Wilkins, 1972) and their volume regulatory ability determined as follows. Suspensions of coelomocytes with a final density of 10,000–20,000 cells/ml were made in approximately 20 ml of ASW of the appropriate osmotic pressure. During the experimental period, cell volume was measured repeatedly to within 75 μm^3 with a Coulter Counter (model ZB, Coulter Electronics Inc., Hialeah, FL) equipped with a Coulter Channelyser (model C-1000) using a 100 μm aperture, and calibrated against 18.18-μm-diameter polystyrene beads. The performance characteristics of the Coulter Counter were unaffected by the temperature-salinity combinations used in this study. Mean cell volume (MCV) at each sampling time was calculated from the Channelyser 100-channel volume-frequency distribution consisting of volume measurements on 15,000–25,000 coelomocytes by the following method: $MCV = \Sigma V_i f_i / \Sigma f_i$, where V_i is the volume of the cells in each channel and f_i is the number of cells in the same channel.

Using this procedure, we determined the volume-regulating ability of *G. dibranchiata* coelomocytes in two experiments. Each experiment was repeated three times with three different coelomocyte suspensions collected from two or three worms per suspension. In the first experiment, cell volume was measured in coelomocytes taken from worms adapted to 996 mosm ASW and exposed to 996 mosm (control) or 505 mosm ASW, at either 5° or 20°C. The MCV was determined 0, 2, 4, 12, and 20 min after mixing with the experimental medium. The data were plotted as the mean change in MCV from the 996 mosm control group at zero time (cell volume $\text{change}_t = \text{MCV}_0 - \text{MCV}_t$).

In the second experiment, cell volume was measured in coelomocytes taken from worms adapted to 996 mosm ASW and exposed to 996 mosm (control) or 592 mosm ASW maintained at 2° or 15°C. The cell volume was monitored for 2 hr, with determinations of MCV at 0, 2, 4, 10, 20, 60, and 120 min after mixing with the experimental medium. After the 120 min measurement was completed, the 592 mosm-2°C cells were warmed to 15°C. These cells were then incubated 20 min longer at 15°C and the MCV determined as above. The data were plotted as the mean change in MCV as above.

Statistical significance was determined by analysis of variance, and significant treatment means were identified using the Student-Newman-Keuls multiple range test.

Red coelomocyte volume regulation: cell volume in acclimated cells

Glycera were acclimated to 1000 or 755 mosm ASW for 14 days. Following the acclimation period, the coelomocytes were collected separately from 14–15 worms in each acclimation salinity. The red coelomocytes were isolated from other coelomic cell types as described above, with all wash and resuspension media at the osmotic concentration of the acclimation medium. The MCV of the red coelomocytes from each worm was then determined by suspending the cells in 20 ml of ASW at the osmotic concentration of acclimation and measuring the cell volume of 15,000–25,000 cells with the Coulter Counter and Coulter Channelyser. The volume regulatory capacity of these acclimated coelomocytes was estimated by suspending cells from each worm adapted to 1000 mosm ASW in 20 ml of 755 mosm ASW. After 3 min, which allows the coelomocytes to attain a near-maximum volume, the MCV was determined as above.

Red coelomocyte amino-acid efflux during acute hypoosmotic stress

Red coelomocytes were isolated from worms acclimated to 1000 mosm ASW as described above. Using the methods described above for measuring cell volume regulation during acute stress, a small sample from each batch of coelomocytes (3.0×10^7 to 7.0×10^7 cells/batch) was tested to establish a positive volume regulatory response to 592 mosm ASW. Cell batches that did not display a positive response were not used in this experiment. Following this preliminary test, half of the cells from each batch were suspended in 2.0 ml of either 592 mosm or 996 mosm ASW. The cells were incubated for 40 min at 15°C with agitation every 5 min. During the incubation period, quadruplicate 10 μl samples were removed from each tube for determination of number by the Coulter Counter. At the end of the incubation period, the cells were centrifuged at $1000 \times g$ for 5 min, and the supernatant analyzed for amino acids on the amino acid analyzer. Incidental hemolysis was determined by converting the hemoglobin in the supernatant to cyanmethemoglobin (Eilers, 1967) and measuring absorbance spectrophotometrically at 540 nm. The red coelomocyte pellet was then hemolysed in distilled water, and samples analyzed for amino acid and hemoglobin concentrations. All amino acid values presented are corrected for the small amount of hemolysis which occurred in both high and low osmotic concentration media. Statistically significant salinity-dependent changes in amino acid efflux were identified by a paired *t* test (Steel and Torrie, 1960).

RESULTS

Coelomic fluid osmotic concentration and body wall tissue water

The osmotic concentration of *G. dibranchiata* coelomic fluid varies directly with the osmotic concentration of the acclimation media over the range of salinities tested (Fig. 1). Although the slope of the regression line describing this relationship ($\pi_{\text{CF}} = 1.005\ (\pi_{\text{MED}}) + 4.8$ mosm where CF = coelomic fluid and MED = medium) is not significantly different from 1.0 ($P > 0.3$), the coelomic fluid is not isosmotic with the environment. Rather, the osmotic concentration is significantly higher ($P < 0.01$) than the environment at every point, averaging 8 mosm hyperosmotic to the acclimation medium.

Body-wall-tissue water, as a percent of wet weight, varied by 12.6% over the range of acclimation salinities tested (Table I).

Body wall and coelomocyte intracellular free amino acids

The intracellular FAA pool of both body wall and coelomocytes decreased significantly ($P < 0.005$) with lowered external salinity (Figs. 2, 3). Both the

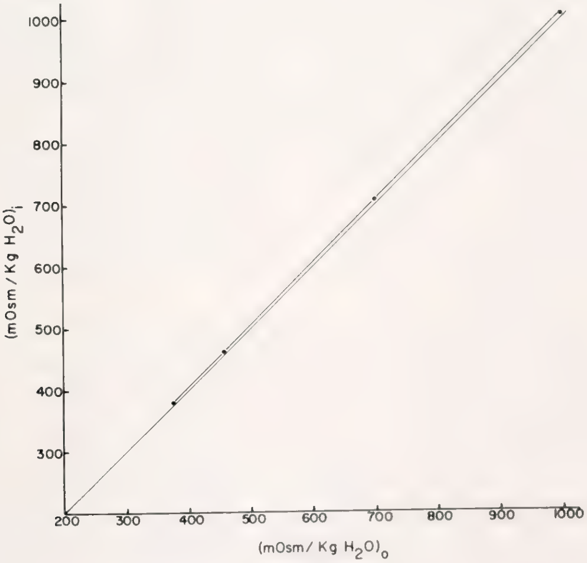


FIGURE 1. Osmotic concentration of *Glycera dibranchiata* coelomic fluid from worms acclimated to 1001, 697, 463, 374, and 230 mosm/Kg H_2O ASW. The points represent the mean osmotic concentration measured from five animals at each salinity. The lower limit of the 99% confidence belt (not shown) about the regression line is hyperosmotic to isosmotic over the entire range of salinities tested.

TABLE I

Body-wall-tissue water from specimens of Glycera dibranchiata *acclimated to reduced salinity. $N = 5$ in each case.*

Acclimation osmotic concentration (mosm/Kg H_2O)	Body-wall-tissue water $\left(\frac{\text{wet wt-dry wt}}{\text{wet wt}} \times 100\ (\pm\text{SE})\right)$
1001	70.6 (±0.34)
697	75.5 (±0.09)
463	80.4 (±0.61)
374	83.2 (±0.70)

composition of the FAA pool, and the amino acids which changed most drastically as a function of the salinity, were different in the two tissues. In the body wall of worms acclimated to 1001 mosm ASW, asparagine, alanine, serine, threonine, and hypotaurine account for 91% of the FAA pool; taurine was less than 2% of the total and proline undetectable. As the external salinity was decreased, the concentrations of asparagine, alanine, serine, and threonine decreased sharply (Fig. 2) while hypotaurine concentration (mean = 78.9 μmol/g dry wt) was independent of salinity change.

In contrast, the coelomocyte FAA pool in 1001-mosm acclimated worms consisted mainly of taurine, proline, asparagine, alanine, glutamine, and serine (Fig. 3). Taurine and proline together accounted for 54% of the 1001-mosm coelomocyte FAA pool. Among the six amino acids composing the bulk of the 1001-mosm FAA pool, proline, taurine, alanine, and glutamine decreased significantly ($P < 0.05$) with decreased acclimation salinity. Asparagine and serine did not change significantly in a salinity-dependent manner. Hypotaurine content, <20 nmol/10^6 cells, was too low to be important as osmotically active solute.

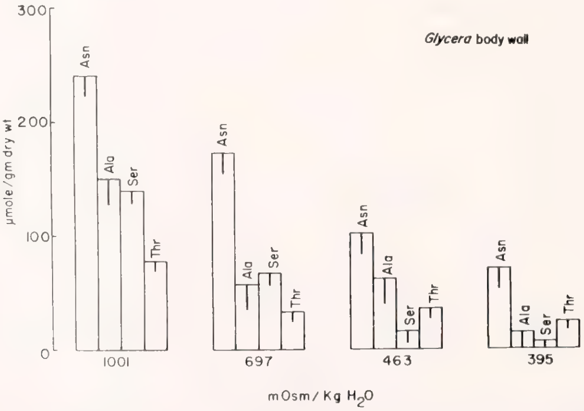


FIGURE 2. Body wall intracellular free amino acid pool of *Glycera dibranchiata* acclimated to 1001, 697, 463, and 395 mosm/Kg H_2O ASW. The values presented are the mean amino acid content measured from five animals at each salinity. Error bars indicate 1 SE.

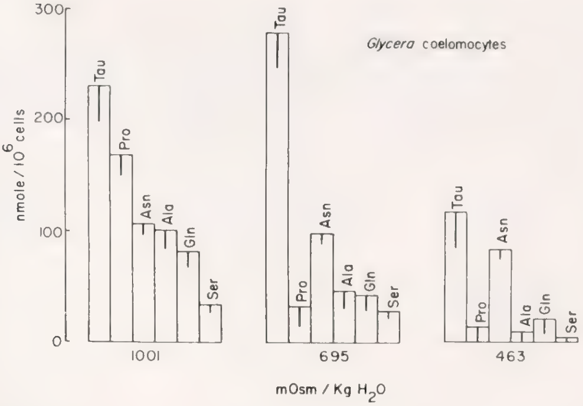


FIGURE 3. Intracellular free amino acid pool of red coelomocytes taken from *Glycera dibranchiata* acclimated to 1001, 695, and 463 mosm/Kg H_2O ASW. The values presented are the mean amino acid content measured from five animals at each salinity. Error bars indicate 1 SE.

Red coelomocyte volume regulation: the acute response

The osmotic swelling of *Glycera* coelomocytes in response to dilution of medium is very rapid. In fact, it is complete within the first 2 min of exposure at 15°–20°C (Figs. 4A, C). Low temperature reduces the rate of osmotic swelling and at 2°–5°C swelling does not reach a maximum for 4–10 min (Figs. 4B, D). The extent of cellular volume increase is a function of both temperature and osmotic gradient. Coelomocytes (mean $MCV_0 = 2344\ \mu m^3$, s.e. $= 80\ \mu m^3$, $n = 3$ coelomocyte suspensions) exposed to a 491 mosm hypoosmotic gradient ($996 \rightarrow 505$ mosm) (Figs. 4A, B) swelled 1632 μm^3 whereas cells exposed to the same osmotic gradient at 20°C swelled only 1333 μm^3. When cells (mean $MCV_0 = 2301\ \mu m^3$, s.e. $= 51\ \mu m^3$, $n = 3$ coelomocyte suspensions) were exposed to a 404 mosm gradient ($996 \rightarrow 592$ mosm) (Figs. 4C, D) the maximum swelling was approximately 1200 μm^3 at both 2° and 15°C. However, the 15° cells reached 1200 μm^3 within 2 min while the 2°C cells required 10 min to reach the same volume. Temperature had no statistically significant effect on coelomocyte volume when the cells from either experiment were exposed to 996 mosm ASW, the salinity of acclimation.

The coelomocyte volume increase in response to hypoosmotic stress was followed by a substantial and continuous reduction in MCV over the remainder of the experiment. This volume recovery was dependent on the magnitude of the osmotic gradient and was reduced by low temperature. The coelomocytes exposed to 505 mosm ASW at 20°C (Fig. 4A) recovered about 319 μm^3 from peak swelling to a final volume 1034 μm^3 greater than the original MCV. When this experiment was conducted at 5°C, the volume recovery was reduced to 190 μm^3 (still significant at $P < 0.05$), resulting in a final volume 1423 μm^3 greater than the original MCV. This pattern was repeated in the second experiment, with coelomocytes exposed to 592 mosm ASW: at 2°C no significant cell volume recovery occurred over 120 min of the experiment (Fig. 4D). Coelomocytes incubated at 15°C recovered 321 μm^3 of cell volume to a final (120 min) volume 920 μm^3 greater than the original MCV (Fig. 4C).

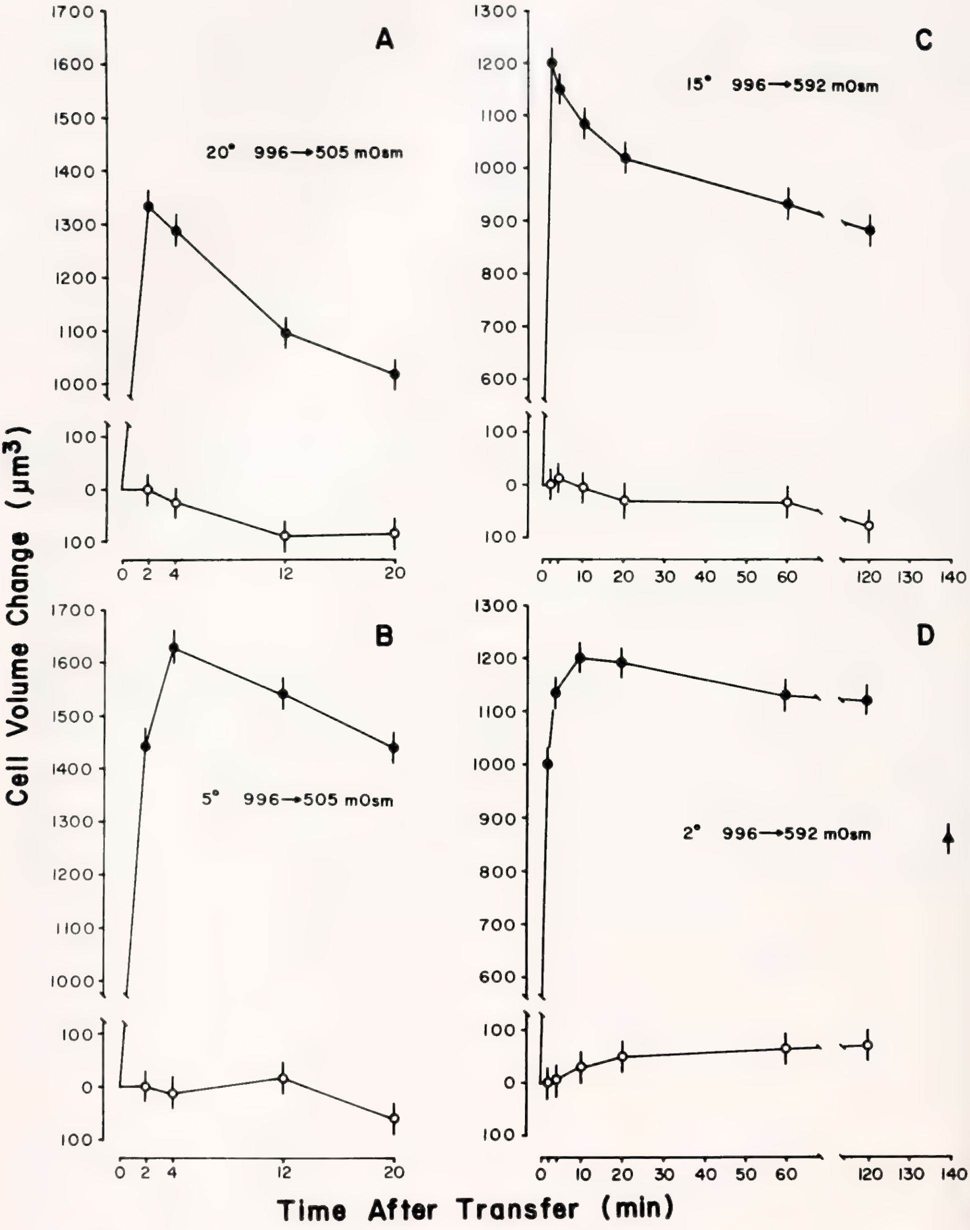


FIGURE 4. *Glycera dibranchiata* red coelomocyte volume changes during acute exposure to hypoosmotic media. A and B show the change in MCV from time zero for coelomocytes exposed to 505 mosm ASW (solid circles) or 996 mosm ASW (open circles) at 20°C (A) or 5°C (B). C and D show the change in MCV from time zero for coelomocytes exposed to 592 mosm ASW (solid circles) or 996 (open circles) at 15°C (C) or 2°C (D). The solid triangle in D is the volume resulting from the return of cells incubated at 2°C and 592 mosm (120 min) to 15°C for 20 min. Points on all graphs are means ± 1 SE (bars).

The low temperature effects were completely reversible. When coelomocytes exposed to 592 mosm ASW at 2°C for 120 min were then warmed to 15°C, these cells recovered to a cell volume 824 μm^3 greater than the original MCV

TABLE II

Cellular volume of Glycera dibranchiata ***red coelomocytes isolated from worms acclimated to 1000 or 755 mosm ASW. Included are values for coelomocytes from the worms acclimated to 1000 mosm ASW and exposed to 755 mosm ASW for 3 min before measurement.***

Acclimation osmotic concentration (mosm/Kg H_2O)	Incubation medium osmotic concentration (mosm/Kg H_2O)	$\overline{MCV}$ (μm^3 ($\pm$SE))	n	Range
1000	1000	2881 (±131)	14	3526–2017
1000	755	4057 (±157)	11	4706–2903
755	755	3378 (± 93)	15	4123–2802

during the following 20 min (Fig. 4D). This was only 93 μm^3 less than the volume of cells incubated at 15°C for 120 min (Fig. 4C).

Red coelomocyte volume regulation: cell volume in acclimated cells

The mean MCV ($\overline{MCV}$) of red coelomocytes from *G. dibranchiata* adapted to 755 mosm for 14 days was significantly greater ($P < 0.05$) than from worms adapted to 1000 mosm ASW (control) (Table II). The $\overline{MCV}$ of the coelomocytes from the 755-mosm-adapted worms was 679 μm^3 lower than cells taken from 1000-mosm-adapted animals and measured following 3 min of exposure to 755 mosm ASW.

Red coelomocyte amino-acid efflux during acute hypoosmotic stress

Hypoosmotic cell volume regulation by *Glycera dibranchiata* coelomocytes is accompanied by an efflux of intracellular amino acids (Fig. 5). Proline efflux was most affected by the decreased medium osmotic pressure, increasing from 1.2 nmol/10^6 cells in 996 mosm ASW to 17.9 nmol/10^6 cells in 592 mosm ASW. Taurine efflux also increased significantly ($P < 0.02$), from 1.9 to 5.4 nmol/10^6

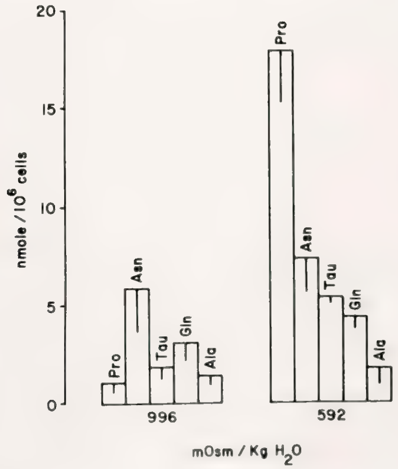


FIGURE 5. Amino acid efflux from *Glycera dibranchiata* red coelomocytes incubated for 40 min in either 996 mosm (control) or 592 mosm ASW. Points are the mean of four cell batches. Error bars indicate 1 SE.

cells, as a result of transfer to 592 mosm ASW. Asparagine, glutamine, and alanine were also present in the efflux (Fig. 5), however, none increased significantly as a consequence of exposure to decreased osmotic pressure.

Discussion

Glycera dibranchiata is an osmoconformer over the 627 mosm range of salinities examined in this study. These results are in general agreement with previous observations (Machin, 1975) except that Machin found the coelomic fluid to be isosmotic to the acclimation media while our results indicate that the coelomic fluid is significantly, though only slightly, hyperosmotic to the acclimation media. Slightly hyperosmotic coelomic fluid or hemolymph has been reported in a number of osmoconformers, polychaetes as well as several other invertebrate groups (see Oglesby, 1973, for an extensive discussion). Although it lacks extracellular osmoregulatory mechanisms, *G. dibranchiata* is very tolerant of changes in salinity. Cell volume regulation in response to a decrease in the osmotic concentration of the extracellular fluid is an important adaptation contributing to the extreme euryhalinity of this osmoconformer. Even our rather imprecise measurement of tissue water, showing only a 12.6% increase in tissue water content during the 2.7-fold dilution of the extra-cellular fluid, indicates effective volume regulation at the cellular level. More direct evidence of effective volume regulation was shown by our measurements of red coelomocyte volume during exposure to dilute media.

Volume regulation in *Glycera* coelomocytes, first described by Machin and O'Donnell (1977), follows the general scheme proposed by Lange and Mostad (1967): a very rapid initial osmotic swelling followed by, or under some conditions reduced by, a volume decrease. Our experiments partially inhibiting the volume regulation with cold demonstrate that the regulatory decrease of the red coelomocyte volume begins before the cells have swollen to the maximum determined by the transmembrane osmotic gradient. For example, red coelomocytes transferred to 505 mosm ASW at 5°C swelled to a greater size, and volume was regulated less effectively, than when the experiment was conducted at 20°C. Further, these experiments indicate that the coelomocytes have started to volume regulate during the first 2 min of exposure to hypoosmotic stress, before the swelling process is complete. Probably the same phenomena were operative when the coelomocytes were transferred to 592 mosm ASW at 2°C, although the data do not show it. The interactions among temperature, osmotic gradient, and physical changes in the structure of water occurring at low temperature are quite complicated, and hence these results are not easily interpreted without further study.

Cell volume is regulated in *Glycera dibranchiata* during hypoosmotic stress by adjustments in the intracellular concentration of FAA, a mechanism common to a number of euryhaline osmoconforming polychaetes (Clark, 1968; Freel *et al.*, 1973). The amino acids used are tissue specific: asparagine, alanine, serine, and threonine account for the solute change in the body wall of acclimated worms; proline, taurine, and, to a lesser extent, alanine and glutamine for the solute changes in red coelomocytes from acclimated worms. The mechanism regulating the intracellular amino acid adjustment is not yet clear. However, the initial volume reduction in the red coelomocytes appears to be accomplished by an efflux of proline and taurine. Further, a comparison of the composition of the amino acid efflux with that of the intracellular amino acid pool indicates that the efflux results from a rather selective change in the permeability of the coelomocyte membrane to particular amino acids in response to low salinity stress.

A comparison of the amino acid concentrations (efflux and cells) in the acute experiments with those in cells of adapted worms shows a difference unaccounted for in our experiments. Possibly, volume regulation by *G. dibranchiata* red coelomocytes under hypoosmotic stress is accomplished in two phases. The first phase, complete within hours, is a rapid loss of solute, which we have detected as FAA efflux. This efflux results in immediate prevention of cell swelling and subsequent rapid volume recovery. The second, slower, phase reduces the intracellular FAA concentration to the level found in acclimated cells and thereby produces a more complete recovery. The means by which the amino acid concentrations are reduced after the initial efflux is unknown, although the likely explanation is some combination of the three usual possibilities, namely, (1) the net amino acid efflux may continue, albeit at a much reduced rate until the total adjustment is complete; (2) the amino acids may be metabolically degraded, or (3) the FAA may be incorporated into protein.

The ubiquity of cell volume regulation mediated, at least in part, by the control of intracellular FAA concentration is well established in invertebrate cells and increasingly so in cells of vertebrates. In spite of this ubiquity, the underlying control mechanisms have been specified only infrequently. The red coelomocytes of *G. dibranchiata* seem to be an excellent preparation for further study of the processes responsible for FAA-mediated cell-volume control during osmotic stress. They provide a readily obtainable, homogeneous source of single cells which survive substantial changes in external osmotic concentration by volume regulation involving a large FAA pool. Perhaps more important, these cells provide the potential for separating regulatory events occurring in the cell membrane from the metabolic functions in the cytoplasm. Using red coelomocytes, we have isolated a cell-free fraction of plasma-membrane vesicles that is currently being used to describe the response of the membrane to hypoosmotic stress.

ACKNOWLEDGMENT

Computer facilities and funds used in this work were supplied by the University of Maryland Computer Science Center.

LITERATURE CITED

AMENDE, L. M., AND S. K. PIERCE, JR., 1978. Hypotaurine: The identity of an unknown ninhydrin-positive compound co-eluting with urea in amino acid extracts of bivalve tissue. *Comp. Biochem. Physiol.* **59**: 257–261.

AMENDE, L. M., AND S. K. PIERCE, 1980. Free amino acid mediated volume regulation of isolated *Noetia ponderosa* red blood cells: Control by Ca^{2+} and ATP. *J. Comp. Physiol.,* **138**: 291–298.

CLARK, M. E., 1968. A survey of the effect of osmotic dilution on free amino acids of various polychaetes. *Biol. Bull.,* **134**: 252–260.

EILERS, R. J., 1967. Notification of final adoption of an international method and standard for hemoglobinometry: specifications for preparation of standard solution. *Am. J. Clin. Pathol.,* **47**: 212–216.

FREEL, R. W., S. G. MEDLER, AND M. E. CLARK, 1973. Solute adjustments in the coelomic fluid and muscle fibers of a euryhaline polychaete, *Neanthes succinea,* adapted to various salinities. *Biol. Bull.,* **144**: 289–303.

GERARD, J. F., AND R. GILLES, 1972. Modification of the amino acid efflux during the osmotic adjustment of isolated axons of *Callinectes sapidus*. *Experientia.*, **28**: 863–864.

GILLES, R., AND E. SCHOFFENIELS, 1969. Isosmotic regulation in surviving nerves of *Eriochier sinensis* during osmotic stress. *Comp. Biochem. Physiol.*, **31**: 927–939.

HOFFMANN, R. J., AND C. P. MANGUM, 1970. The function of coelomic cell hemoglobin in the polychaete *Glycera dibranchiata*. *Comp. Biochem. Physiol.*, **36**: 211–228.

LANG, M. A., AND H. GAINER, 1969a. Volume control by muscle fibers of blue crab. *J. Gen. Physiol.*, **53**: 323–341.

LANG, M. A., AND H. GAINER, 1969b. Isosmotic intracellular regulation as a mechanism of volume control in crab muscle fibers. *Comp. Biochem. Physiol.*, **30**: 445–456.

LANGE, R., AND A. MOSTAD, 1967. Cell volume regulation in osmotically adjusting marine animals. *J. Exp. Mar. Biol. Ecol.*, **1**: 209–219.

MACHIN, J., 1975. Osmotic responses of the bloodworm, *Glycera dibranchiata* Ehlers: A graphical approach to the analysis of weight reduction. *Comp. Biochem. Physiol.*, **52**: 49–54.

MACHIN, J., AND M. J. O'DONNELL, 1977. Volume regulation in the coelomocytes of the blood worm *Glycera dibranchiata*. *J. Comp. Physiol.*, **117**: 303–311.

OGLESBY, L. C., 1973. Salt and water balance in lugworms (Polychaeta: Arenicolidae), with particular reference to *Abarenicola pacifica* in Coos Bay, Oregon. *Biol. Bull.*, **145**: 180–199.

PIERCE, S. K., JR., AND M. J. GREENBERG, 1972. The nature of cellular volume regulation in marine bivalves. *J. Exp. Biol.*, **57**: 681–692.

PIERCE, S. K., JR., AND M. J. GREENBERG, 1973. The initiation and control of free amino acid regulation of cell volume in salinity stressed marine bivalves. *J. Exp. Biol.*, **59**: 435–446.

SEAMONDS, B., AND H. R. SCHUMACHER, JR., 1972. Fine structure of erythrocytes of the common bloodworm *Glycera dibranchiata*. *Cytologica.*, **37**: 359–363.

SNEDECOR, G. W., AND W. G. COCHRAN, 1967. *Statistical Methods.* Ames, Iowa: The Iowa State Univ. Press.

STEEL, R. G. D., AND J. H. TORRIE, 1960. *Principles and Procedures of Statistics.* New York: McGraw-Hill.

WATTS, J. A., AND S. K. PIERCE, 1978. A correlation between the activity of the divalent cation activated adenosine triphosphatase in the cell membrane and low salinity tolerance of the ribbed mussel *Modiolus demissus demissus*. *J. Exp. Zool.*, **204**: 49–56.

WILKENS, L. A., 1972. Electrophysiological studies on the heart of the bivalve mollusc, *Modiolus demissus*. I. Ionic basis of the membrane potential. *J. Exp. Biol.*, **56**: 273–291.

Reference: *Biol. Bull.*, **159**: 639–648. (December, 1980)

IN VITRO CARBON FIXATION BY *PROCHLORON* SP. ISOLATED FROM *DIPLOSOMA VIRENS*

CHARLES R. FISHER, JR., AND ROBERT K. TRENCH

Department of Biological Sciences and the Marine Science Institute, University of California at Santa Barbara, Santa Barbara, California 93106

ABSTRACT

Prochloron sp. isolated from *Diplosoma virens* and incubated in the light in $NaH^{14}CO_3$ demonstrated a high photosynthetic capacity (up to 3.7 $\mu gC\cdot(\mu g$ Chlorophyll $a)^{-1}\cdot hr^{-1}$). *In vitro* these cyanobacteria release a maximum of 7% of the ^{14}C they fix in the light. Dark fixation was found to be maximally 3% of light fixation and release in the dark averaged 26% of the total ^{14}C fixed in the dark. These data imply that the organic carbon released by these cyanobacteria may not be quantitatively important to the host.

The labeled compound released by *Prochloron* in the light is glycolic acid. The major compounds produced by light and dark carbon fixation in *Prochloron* are identified, and similarities to other photosynthetic cyanobacteria are noted.

INTRODUCTION

Prochloron sp. (Lewin, 1976, 1977) is a cyanobacterium that lives in the common cloacal cavity of the colonial didemnid ascidian *Diplosoma virens*. *Prochloron* is prokaryotic with no internal organelles (Whatley, 1977), and possesses a peptidoglycan cell wall containing diaminopimelic and muramic acids, as do the walls of other cyanobacteria (Moriarty, 1979). Unlike many cyanobacteria, *Prochloron* lacks phycobilin pigments and contains chlorophyll (Chl) *a* and uniquely among the cyanobacteria, chlorophyll *b* (Lewin and Withers, 1975; Thorne *et al.*, 1977; Withers *et al.*, 1978). Thinh (1978) and Whatley *et al.* (1979) concluded that the photosynthetic lamellae in *Prochloron* were composed of appressed thylakoids unlike other photosynthetic cyanobacteria, but Thorne *et al.* (1977) do not agree.

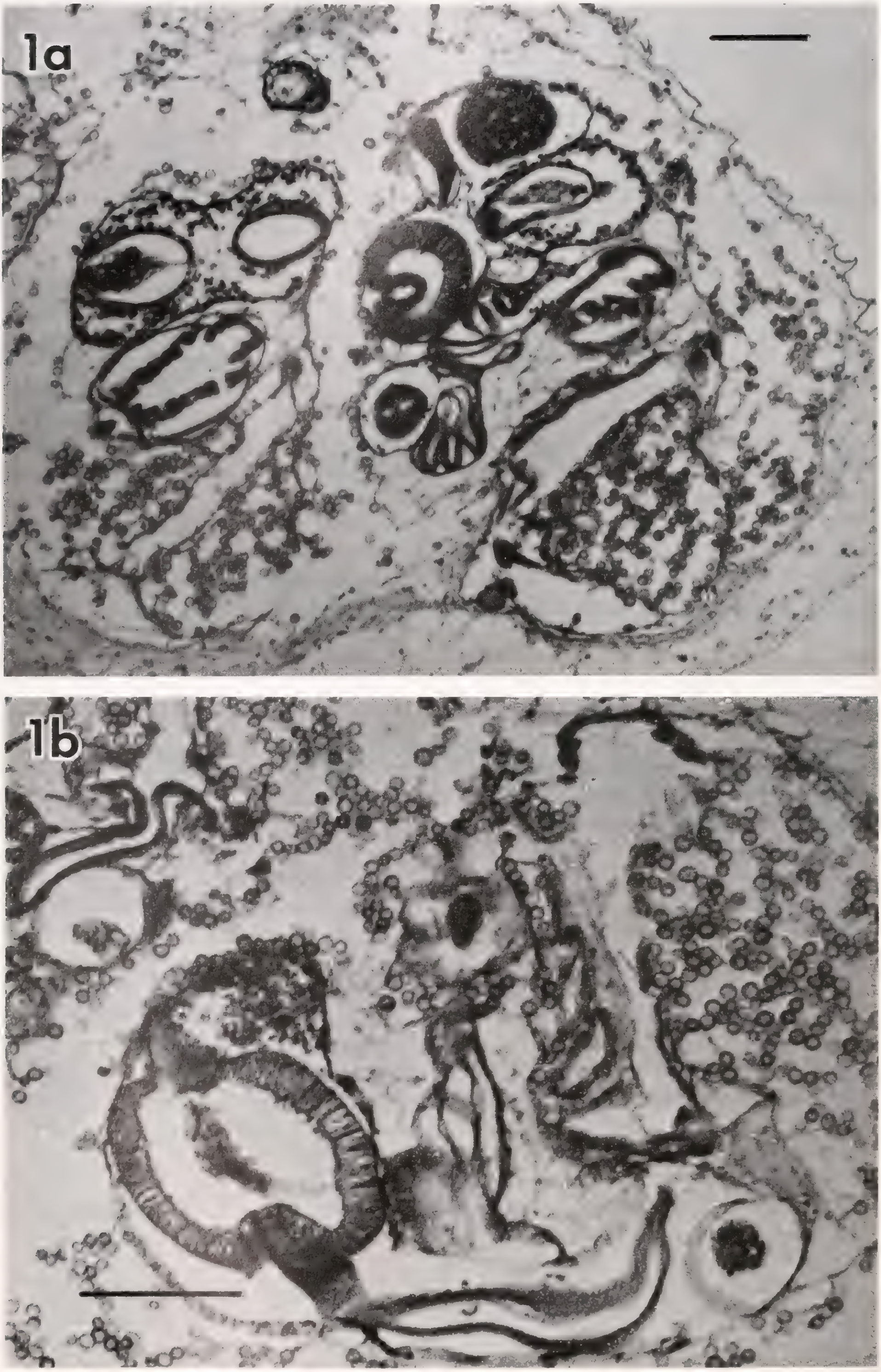
Prochloron has been characterized with respect to its photosynthetic capacity both *in vivo* and *in vitro* (Thinh and Griffiths, 1977) and photosynthetic products *in vivo* (Akazawa *et al.*, 1978). There are no reports of translocation or of the *in vitro* carbon fixation products. This study was undertaken principally to determine the pattern of photosynthetic carbon fixation *in vitro* and to explore the possible release of organic carbon.

MATERIALS AND METHODS

Collection of organisms and isolation of Prochloron

The colonial ascidians *Diplosoma virens* containing *Prochloron* were collected living epizoically on the bubble alga *Dictyosphaera* sp. from "fish pond reef"

Received May 2, 1980; accepted September 1, 1980.


1a
1b

in Kaneohe Bay, Oahu, Hawaii. The animals were maintained in running sea water tanks for a maximum of 4 hr before use.

Small pieces of *Dictyosphaera* were placed in 400 ml of 0.05 M HEPES-buffered sea water (pH 8.1) for 20 min before isolation of the cyanobacteria. This was done in order to mitigate the possible adverse effects of acid released from the animals during the isolation procedure. *Prochloron* cells were isolated by gently stroking the animals with a syringe while pulling up on the plunger. The cyanobacteria were concentrated by gentle centrifugation, washed three times in filtered sea water and finally suspended in filtered sea water at concentrations between 1 and 5 × 10^6 cells/ml. Sea water used in these studies was filtered through Millipore filters of 0.22 μm porosity.

Incubations with $NaH^{14}CO_3$

Small test tubes mounted on a floating plexiglass rack were used as incubation vessels. For dark incubations, the test tubes were wrapped in two layers of electrician's tape and covered with aluminum foil. For all incubations, 2 ml of cell suspension was allowed to equilibrate for at least 15 min in the incubation vessel at 25°C before the $NaH^{14}CO_3$ was added.

In early experiments, $NaH^{14}CO_3$ was added to a concentration of 1.0 μCi/ml. In later experiments (those destined for chromatographic analyses) $NaH^{14}CO_3$ was added to a concentration of 25 μCi/ml. After addition of the $NaH^{14}CO_3$, aliquots were removed for determination of initial activity. At the end of an experiment, or whenever samples were taken, the cyanobacteria were separated by centrifugation, and hot 95% ethanol was separately added to the pellets and the supernatants in order to stop further biochemical activity. All incubations were of 1 hr duration. All experiments were conducted between 1000 and 1400 hr and the ambient illumination varied with cloud cover from 300 to 2000 μ Einsteins$\cdot m^{-2} \cdot sec^{-1}$.

Estimation of chlorophyll

Samples of 5 ml of cell suspensions were centrifuged at 2800 RPM for 3 min and the supernatant discarded. The pellets were frozen and thawed three times in succession and then extracted in 90% cold acetone. Absorbances were read at 664 and 647 nm in a Beckman DBG spectrophotometer, and chlorophyll estimated using the equations of Jeffrey and Humphrey (1975).

Estimation of cell densities

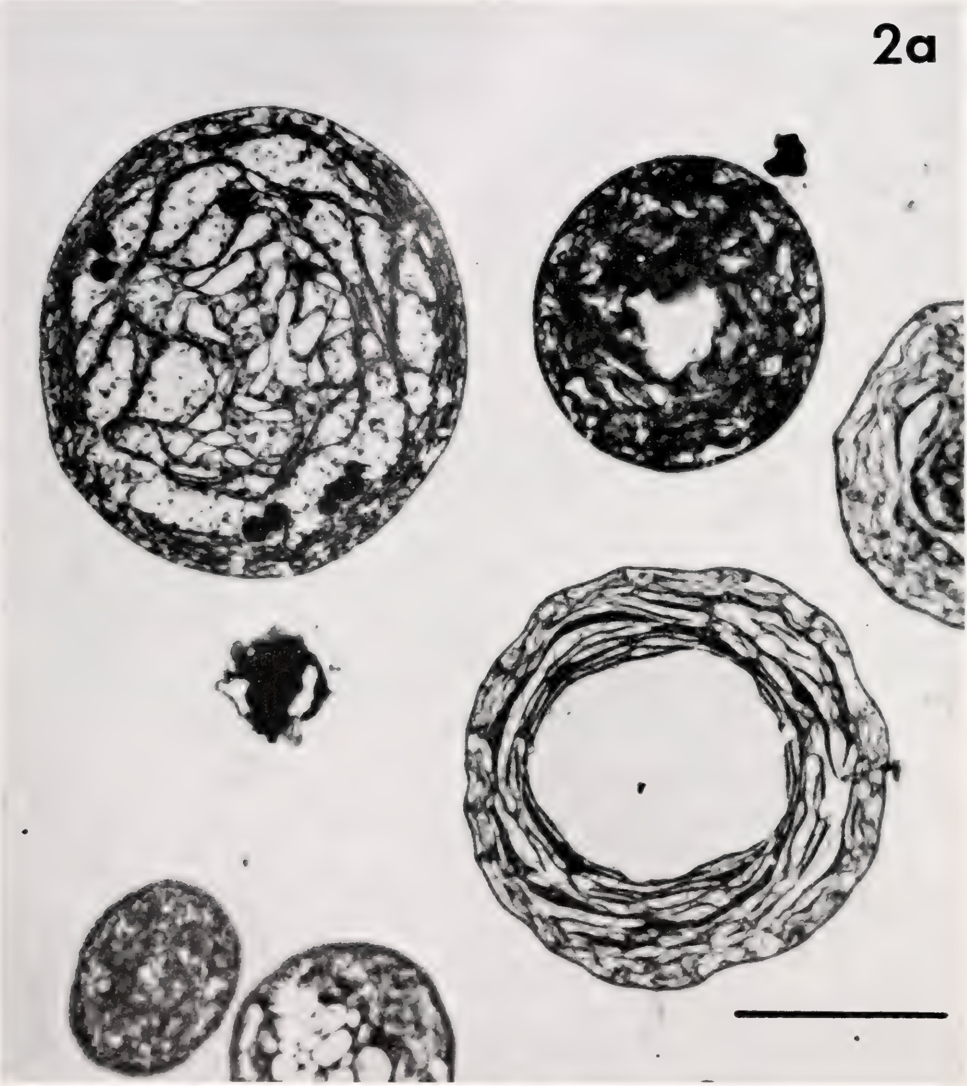
Cell concentrations were estimated using a Spencer Bright Line Haemocytometer. There was probably a large inherent error due to the tendency of the cells to form clumps. Five replicate counts were made for each estimate.

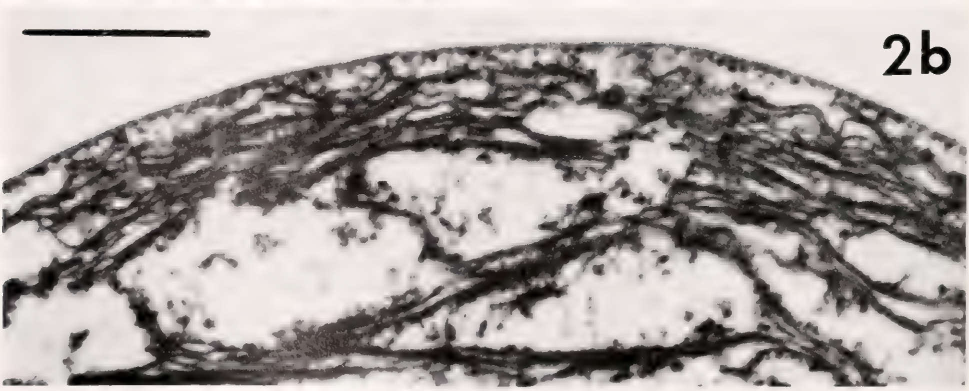
Assay of radioactivity

Aliquots of samples were acidified with 3 M HCl, heated to 90°C and repeatedly purged with a stream of air for 30 min to remove inorganic carbon. The samples

---

FIGURE 1. a. Light micrograph of paraffin-embedded section of *D. virens*, showing the distribution of *Prochloron* in the cloacal cavity. Magnification appx. 140 ×. Scale bar = 100 μm. b. Light micrograph of paraffin-embedded section of *D. virens* showing a closer view of the cloaca. Magnification appx. 230 ×. Scale bar = 100 μm.


2a


2b

were neutralized with 3 M NaOH. Samples of 0.1 ml were then placed in plastic scintillation vials with 10 ml of Aquasol, and the radioactivity measured on a Beckman liquid scintillation spectrometer. Corrections were made for background and quenching.

Chromatographic analyses

The labeled cyanobacteria were extracted in hot 90% ethanol. Particulate matter was removed by centrifugation. The incubation media containing organic ^{14}C was evaporated to dryness in a Buchler Evapomix and the organic carbon extracted from the salt in absolute ethanol (Trench, 1971b).

Cell extracts and media were applied onto 46 × 57 cm sheets of Whatman #4 chromatography paper, and the compounds separated by two-dimensional chromatography with phenol: water (25:10, w/v) in the first dimension and n-butanol: propionic acid: water (623:60:437, v/v/v) in the second dimension (Bassham and Calvin, 1957). Radioactive compounds were detected by autoradiography using Kodak single-coated blue-sensitive medical X-ray film.

The separated radioactive compounds were identified through co-chromatography (finger-printing) with authentic substances in different solvent systems, on Whatman #1 paper and thin-layer plates coated with Silica Gel-G. Sugars were visualized with the silver nitrate reaction (Trevelyan *et al.,* 1950) and amino acids with ninhydrin (Toennies and Kolb, 1951).

Sugar phosphates were dephosphorylated with phosphatase (Sigma Chemical Corp., St. Louis, Mo.), and the pure sugars identified by co-chromatography. Polyglucans were hydrolyzed with 3 M HCl at 100°C, and the products identified by co-chromatography. The ethanol insoluble residue was not analyzed.

RESULTS

The "green" cyanobacterium *Prochloron* is an extra-cellular symbiont occurring in the common cloacal cavities of the colonial didemnid ascidian *Diplosoma virens* (see Figs. 1a, b). The ultrastructure of *Prochloron* (Figs. 2a, b) indicates a typical cyanobacterial morphology, with the exception of the thylakoid arrangement. Thinh (1978) and Whatley *et al.* (1979) emphasize the tendency towards stacked thylakoids, implying a significant similarity to the thylakoid arrangement in chloroplasts of green algae (see also Giddings *et al.,* 1980). However, Thorne *et al.* (1977) do not concur with this view, and based on our observations (Figs. 2a, b) and those originally reported by Whatley (1977), the thylakoid arrangement in *Prochloron* sp. is highly variable.

With illumination between 300 and 2000 $\mu E \cdot m^{-2} \cdot sec^{-1}$, isolated *Prochloron* photoassimilated carbon at rates ranging from 0.67 to 3.72 $\mu gC \cdot (\mu g\ Chl\ a)^{-1} \cdot hr^{-1}$. Heterotrophic dark fixation rates never exceeded 0.038 $\mu gC \cdot (\mu g\ Chl\ a)^{-1} \cdot hr^{-1}$, and maximally represented 3% of light fixation (Table I).

The rates of photosynthetic carbon assimilation reported here are higher than those reported by Akazawa *et al.* (1978) for the intact association and are in good agreement with the rates reported by Thinh and Griffiths (1977) for the intact association as well as isolated cyanobacteria.

FIGURE 2. a. Electron micrograph of *Prochloron in situ* in *D. virens.* Magnification appx. 5000 ×. Scale bar = 5 μm. b. Electron micrograph of *Prochloron* showing details of stacked and unstacked thylakoids. Magnification appx. 24,000 × Scale bar = 1 μm.

TABLE I

Quantification of acid stable ^{14}C found in cells and media after 1 hr incubations. Note: 2a and 4a were run simultaneously with 2 and 4, but were incubated in the presence of host homogenate.

Experiment No.	μgC fixed $\cdot$ (μg Chl a)$^{-1} \cdot$ hr^{-1}		μgC released $\cdot$ (μg Chl a)$^{-1} \cdot$ hr^{-1}		Dark fixation (% light fixation)	Minimum light intensity μE $\cdot$ m$^{-2} \cdot$ sec^{-1}
	Light	Dark	Light	Dark		
2	0.702	0.008	0.0042	0.0047	1.1	300
2a	0.680	0.020	0.0095	0.0030	3.0	300
4	0.800	0.008	0.0088	0.0027	1.0	300
4a	0.670	0.011	0.0087	0.0042	1.6	300
6	0.730	0.015	0.0175	0.0020	2.1	300
7	1.000	0.014	0.0720	0.0011	1.4	500
8	3.720	0.022	0.1116	0.0044	0.6	500
9	3.300	0.029	0.1782	0.0072	0.9	750
10	2.750	0.038	0.0935	0.0082	1.4	1000

From Table I it is apparent that the maximum quantity of photosynthetically fixed carbon released was 0.178 μgC $\cdot$ (μg Chl a)$^{-1} \cdot$ hr^{-1}, representing 5.4% of the total carbon fixed. Release of photosynthate by *Prochloron* was not enhanced by incubation in the presence of a homogenate of tissues of the ascidian host, even in buffered media (Table I, Experiments 2a, 4a). This is in contrast to symbiotic dinoflagellates (Muscatine, 1967; Muscatine *et al.*, 1972; Trench, 1971b, c; 1974).

The only ^{14}C-labeled compound released by *Prochloron* in sufficient quantity for identification by co-chromatography was glycolic acid (Fig. 3a). The ethanol-soluble photosynthetic products extracted after 1 hr incubation are shown in Figure 3b. Such a pattern is typical of photosynthetic cyanobacteria (Kremer *et al.*, 1979). The primary photosynthetic end products are glucose, maltose, fructose, sugar phosphates, and polyglucans. No labeled sucrose was detected.

Comparison of the patterns of organic ^{14}C found in the media with those found in the cell extracts (*cf.* Figs. 3a, b) indicates that ^{14}C found in the media is due to selective release and not cell lysis.

The primary heterotrophic fixation products of *Prochloron* labeled in the dark were identified as aspartic acid (25%), glutamic acid (50%), and glutamine (20%). The remainder occurred in insufficient quantity for identification (Fig. 4).

DISCUSSION

The photosynthetic cyanobacterium *Prochoron* isolated from the didemnid ascidian *Diplosoma virens* assimilated carbon *in vitro* at a high rate. Earlier attempts (Akazawa *et al.*, 1978) to isolate photosynthetically active *Prochloron* were frustrated by the release of acid by the host (Thorne *et al.*, 1977). In our experiments this difficulty was overcome by performing the isolation in HEPES-buffered sea water. Despite this precaution, the cyanobacteria became photosynthetically inactive within 5 hr, suggesting that some aspect of the host environment is essential to their survival.

Analyses of the media in which the isolated cyanobacteria were incubated showed the presence of ^{14}C-labeled glycolic acid. This indicates, by comparison with the numerous ^{14}C-labeled compounds detected in the cell extract, that the organic carbon detected in the media is the result of selective release and not cell

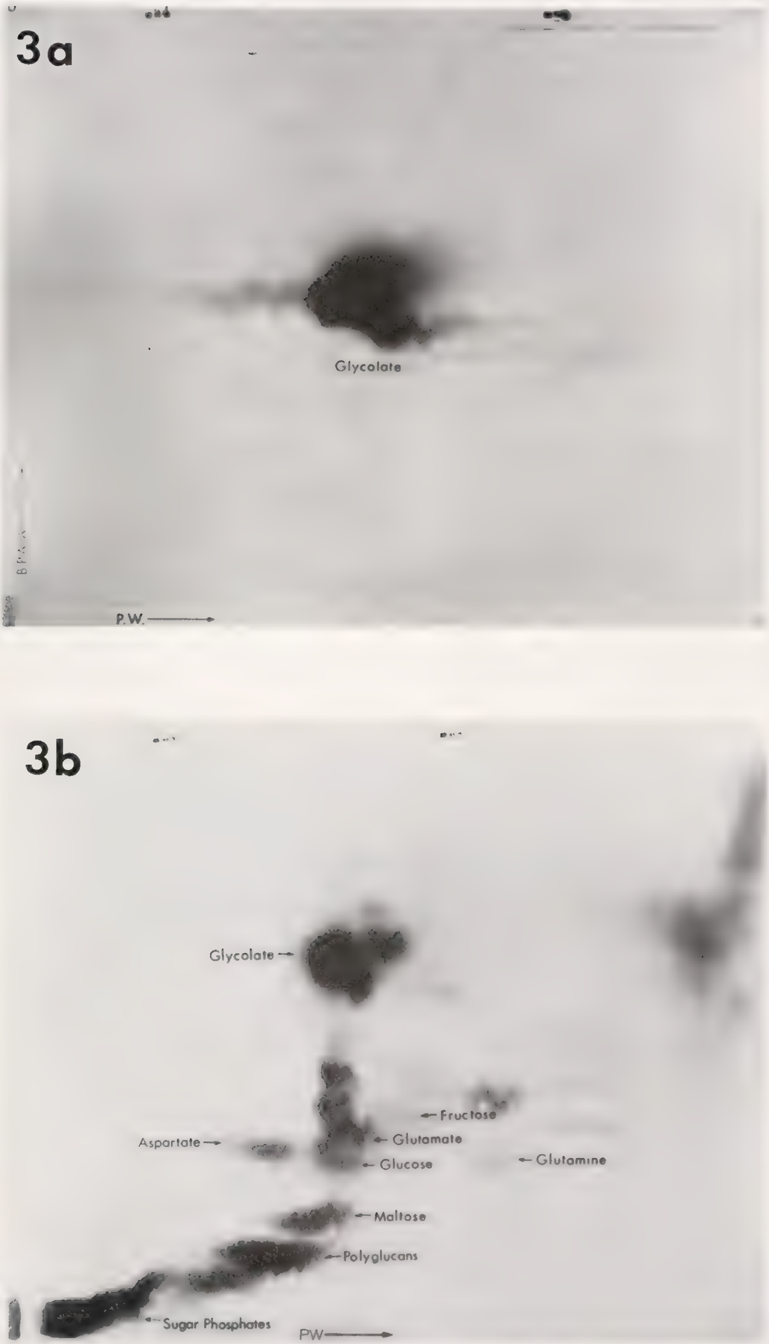


FIGURE 3. a. Two-dimensional radiochromatogram of the medium in which *Prochloron* was incubated in $NaH^{14}CO_3$ in the light. *P.W.*, phenol : water ; *B.P.A.W.*, butanol, propionic acid: water. b. Two-dimensional radiochromatogram of the ethanol extract of the same cells.

lysis. Similarly, the difference between the light and dark fixation patterns indicates that the ^{14}C fixed in the dark controls was a result of heterotrophic dark fixation and not a small amount of photosynthesis due to light leaks in the apparatus.

In other associations involving algae and invertebrate hosts, the *in vitro* release of photosynthate corroborated evidence of *in vivo* translocation of photosynthetically fixed carbon (Muscatine, 1967; Trench, 1971a, b, 1974). Trench (1971c) and (1971c) and Muscatine *et al.* (1972) found that adding homogenates of the tissues of the host animals to suspensions of isolated "zooxanthellae" resulted in an increased release of photosynthetically fixed carbon. In the case of *Prochloron,* the quantity of released carbon never exceeded 0.178 μgC$\cdot$(μg Chl *a*)$^{-1}\cdot$hr^{-1} (or 5.4% of the carbon fixed). By extrapolation, it can be concluded that *if* the cyanobacteria function *in vivo* as they do *in vitro,* then *Prochloron* probably contributes minimally to the carbon metabolism of its hosts.

Several phytoplankters have been shown to release (excrete) from 1 to 17% of the carbon they fix photosynthetically (Hellebust, 1965). Glycolic acid was found to be the major product excreted by *Chlorella pyrenoidosa* (Fogg, 1962) and by several species of *Chlamydomonas* (Lewin, 1957). Therefore, as far as the release of photosynthetic products is concerned, *Prochloron* resembles more closely the free-living phytoplankton than symbiotic algae such as "zooxanthellae" and "zoochlorellae" (see Trench, 1979).

Because of the presence of chlorophyll *b* in *Prochloron,* it has become somewhat popular to speculate that an organism such as *Prochloron* may have been the evolutionary precursor of the chloroplasts of green plants. In fact the presence of chlorophyll *b* prompted Lewin (1976) to propose a new Division for these organisms. The search for other characteristics relating these cells to the Chloro-

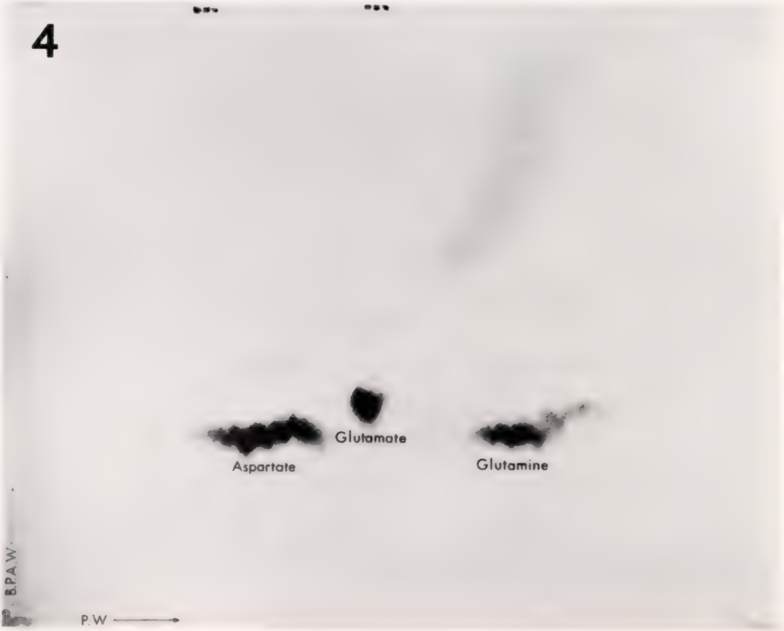


FIGURE 4. Two-dimensional radiochromatogram of ethanol extract of *Prochloron* incubated in NaH^{14}CO$_3$ in the dark.

phyta continues. As far as the patterns of photosynthetic and heterotrophic carbon fixation is concerned, *Prochloron* demonstrates characteristics typical of cyanobacteria (Kremer *et al.*, 1979). The absence of sucrose as a photosynthetic end product also distinguishes *Prochloron* from chlorophytes (Meeuse, 1962).

ACKNOWLEDGMENTS

We wish to thank the Director, Dr. John Caperon, and the faculty and staff of Hawaii Institute of Marine Biology for the use of their facilities. We are grateful to Professor L. Muscatine for his advice and suggestions. Partial support for this work was provided by NSF, PCM78-15209 to R.K.T.

LITERATURE CITED

AKAZAWA, T., E. H., NEWCOMB, AND C. B. OSMOND, 1978. Pathway and products of CO_2-fixation by green prokaryotic algae in the cloacal cavity of *Diplosoma virens*. *Mar. Biol.*, **47**: 325–330.

BASSHAM, J. A., AND M. CALVIN, 1957. *The path of carbon in photosynthesis.* Prentice-Hall, Englewood Cliffs, N. J. 104 pp.

FOGG, G. E., 1962. Extracellular products. Pp. 475–489 in R. A. Lewin, Ed., *Physiology and biochemistry of algae.* Academic Press, New York.

GIDDINGS, T. H. JR., N. W. WITHERS, AND L. A. STAEHELIN, 1980. Supramolecular structure of stacked and unstacked regions of the photosynthetic membranes of *Prochloron* sp., a prokaryote. *Proc. Nat. Acad. Sci. U.S.A.*, **77**: 352–356.

HELLEBUST, J. A., 1965. Excretion of some organic compounds by marine phytoplankton. *Limnol. Oceanog.*, **10**: 192–206.

JEFFREY, S. W., AND G. F. HUMPHREY, 1975. New spectrophotometric equations for determining chlorophylls a, b, c_1, and c_2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanzen.*, **167**: 191–194.

KREMER, P. P., L. KIES, AND A. ROSTAMI-RAKET, 1979. Photosynthetic performance of the cyanelles in the endocyanomes *Cyanophora*, *Glaucosphaera*, *Gloeochaete*, and *Glaucocystis*. *Z. Pflanzenp.* **92**: 303–317.

LEWIN, R. A., 1957. Excretion of glycolic acid by *Chlamydomonas*. *Bull. Jpn. Soc. Phycol.*, **5**: 74–75.

LEWIN, R. A., AND N. W. WITHERS, 1975. Extraordinary pigment composition of a prokaryotic alga. *Nature*, **256**: 735–737.

LEWIN, R. A., 1976. Prochlorophyta as a proposed new Division of algae. *Nature*, **261**: 697–698.

LEWIN, R. A., 1977. *Prochloron*, type genus of the Prochlorophyta. *Phycologia*, **16**: 217.

MEEUSE, B. J. D., 1962. Storage products. Pp. 289–313 in R. A. Lewin, Ed., *Physiology and Biochemistry of Algae.* Academic Press, New York.
120: 191–193.

MORIARTY, D. J. W., 1979. Muramic acid in the cell walls of *Prochloron*. *Arch. Microbiol.*,

MUSCATINE, L., 1967. Glycerol excretion by symbiotic algae from corals and *Tridacna* and its control by the host. *Science*, **156**: 516–519.

MUSCATINE, L., E. CERNICHIARI, AND R. R. POOL, 1972. Some factors affecting selective release of soluble organic material by zooxanthellae from reef corals. *Mar. Biol.*, **13**: 298–308.

THINH, L. V., AND D. J. GRIFFITHS, 1977. Studies of the relationship between the ascidian *Diplosoma virens* and its associated microscopic algae. I. Photosynthetic characteristics of the algae. *Aust. J. Mar. Freshw. Res.*, **28**: 673–681.

THINH, L. V., 1978. Photosynthetic lamellae of *Prochloron* (Prochlorophyta) associated with ascidian *Diplosoma virens* (Hartmeyer) in the vicinity of Townsville. *Aust. J. Bot.*, **26**: 617–620.

THORNE, S. W., E. H. NEWCOMB, AND C. B. OSMOND, 1977. Identification of chlorophyll b in extracts of prokaryotic algae by fluorescence spectroscopy. *Proc. Nat. Acad. Sci. U.S.A.*, **14**: 575–578.

TOENNIES, G., AND J. J. KOLB, 1951. Techniques and reagents for paper chromatography. *Anal. Chem.*, **23**: 823–826.

TRENCH, R. K., 1971a. The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. I. The assimilation of photosynthetic products of zooxanthellae by two marine coelenterates. *Proc. R. Soc. Lond. B. Biol.,* **177**: 225–235.

TRENCH, R. K. 1971b. The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. II. Liberation of fixed ^{14}C by zooxanthellae *in vitro. Proc. R. Soc. Lond. B. Biol.,* **177**: 237–250.

TRENCH, R. K., 1971c. The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. III. The effect of homogenates of host tissues on the excretion of photosynthetic products *in vitro* by zooxanthellae from two marine coelenterates. *Proc. R. Soc. Lond. B. Biol.,* **177**: 251–264.

TRENCH, R. K., 1974. Nutritional potentials in *Zoanthus sociatus. Helgol. Wiss. Meeresunters.,* **26**: 174–216.

TRENCH, R. K., 1979. The cell biology of plant-animal symbioses. *Ann. Rev. Plant Physiol.,* **30**: 485–531.

TREVELYAN, W. F., D. P. PROCTOR, AND J. S. HARRISON, 1950. Detection of sugars on paper chromatograms. *Nature,* **166**: 444–445.

WHATLEY, J. M., 1977. The fine structures of *Prochloron. New Phytol.,* **79**: 309–313.

WHATLEY, J. M., P. JOHN, AND F. R. WHATLEY, 1979. From extracellular to intracellular: The establishment of mitochondria and chloroplasts. *Proc. R. Soc. Lond. B. Biol.* **204**: 165–187.

WITHERS, N., W. VIDAVER, AND R. A. LEWIN, 1978. Pigment composition, photosynthesis, and fine structure of a nonblue-green prokaryotic algal symbiont (*Prochloron* sp.) in a didemnid ascidian from Hawaiian waters. *Phycologia,* **17**: 167–171.

Reference: *Biol. Bull.*, **159**: 649–655. (December, 1980)

THE EFFECTS OF CAFFEINE AND THEOPHYLLINE ON THE PHOTOTACTIC RHYTHM OF *CHLAMYDOMONAS REINHARDII*

JUDITH E. GOODENOUGH[1] AND VICTOR G. BRUCE[2]

[1] *Department of Zoology, University of Massachusetts, Amherst, Mass. 01003, and*
[2] *Department of Biology, Princeton University, Princeton, N. J. 08540*

ABSTRACT

Caffeine and theophylline were found to increase the period length of the phototactic rhythm of *Chlamydomonas reinhardii* in a dose-related manner. Treatment with caffeine resulted in average period increase of 11.2% (3 mM), 16.4% (5 mM), and 29% (10 mM). Theophylline caused smaller period increases than did caffeine: an average period lengthening of 4.2% (3 mM) and 6.1% (5 mM).

INTRODUCTION

Many of the important physiological processes in plants and animals are rhythmic. Therefore, it is of great importance to learn the nature of the clock driving these rhythms. One method of attempting to decipher the mechanism of the clock has been to subject the organism to chemicals with known effects in the hope that the clockworks will be altered in a way that will reveal some aspect of their machinery. If a drug reaches the clock and interferes with some process important to its timekeeping, sustained administration of the drug would be expected to result in a change in the period length of the observed rhythm.

Although we are still far from knowing the molecular basis for circadian rhythmicity, we are beginning to identify some processes that are directly or indirectly involved in the timing mechanism. Translation of proteins on the 80S ribosomes appears to be important to the functioning of the clock. This generalization is based on the observation that inhibitors of protein synthesis on the 70S ribosomes, such as streptomycin and chloramphenicol, usually have no effects on the period or phase of most biological rhythms (Karakashian and Hastings, 1962, 1963; Sweeney *et al.*, 1967; Enright, 1971; Mergenhagen and Schweiger, 1975a) while inhibitors of protein synthesis on the 80S ribosomes, such as cycloheximide, puromycin, and anisomycin, often affect the timing process (Brinkmann, 1971, 1973; Feldman, 1967; Mergenhagen and Schweiger, 1975b; Karakashian and Schweiger, 1976a, b; Rothman and Strumwasser, 1977; Jacklet, 1977; Walz and Sweeney, 1979).

Some primarily circumstantial evidence indicates that membranes are involved in generating circadian oscillations. Substances such as ethanol (Keller, 1960; Bünning and Baltes, 1962; Bünning and Moser, 1973; Brinkmann, 1974, 1976; Sweeney, 1974), K^+ and valinomycin, an ionophore of K^+ (Eskin, 1972; Bünning and Moser, 1973) and Li^+ (Engelmann 1972, 1973; Engelmann *et al.*, 1974; Eskin, 1977) modify rhythms and are also known to alter membrane function.

Received May 22, 1980; accepted September 25, 1980.

Abbreviations: + mating type, mt^+; − mating type, mt^-; high salt-concentration medium, HSM.

A role for cyclic AMP (cAMP) in the functioning of the biological clock has also been postulated (Feldman, 1975; Sweeney, 1976). A reason for this speculation that led to testing the hypothesis chemically is that the enzymes which control the levels of cAMP, adenyl cyclase and cAMP phosphodiesterase, are often membrane-bound. In addition, Cummings (1975) has suggested a more intimate involvement of cAMP in the timing process. He proposes a feedback system in which the levels of cAMP control and in turn are controlled by the activity of adenyl cyclase and phosphodiesterase.

Chlamydomonas reinhardii has a well defined rhythm in phototaxis (Bruce, 1970, 1972). In an attempt to test the speculations that cAMP may be involved in the functioning of the biological clock, we subjected *Chlamydomonas* to inhibitors of cyclic AMP phosphodiesterase and observed the effect on the organisms' rhythm in phototaxis.

Materials and Methods

Culture techniques

Both the + mating type (mt$^+$) and the − mating type (mt$^-$) of strain 137 F of *Chlamydomonas reinhardii* were used in these experiments. The strains were originally obtained from Lutz Wiese in 1960 and have been maintained at Princeton University since then. Experimental cultures were grown on a shaker table under continuous illumination from cool white flourescent lamps (1500–3000 lux) at 22° ± 1°C. The cultures were grown in 100 ml of 0.3 HSM medium (high salt-concentration medium) (Bruce, 1972) in 200 ml Erlenmeyer flasks to a density of approximately 1–2 × 10^6 cells/ml.

Phototactic assay

Phototaxis was assayed on 1.5 ml samples of the culture placed in plastic tissue culture trays (Linbro Plastic) to which the drugs were added. The cells were placed in the apparatus in darkness that was interrupted at 2 hr intervals with 0.5 hr of a vertical light beam. While the light was on, organisms in a positive phototactic state were attracted to the beam and accumulated there, diminishing the light reaching a photocell positioned above each beam. The photocell measured the intensity of transmitted light during the last 10 min the light was on. Thus the light beam not only elicited the phototactic response, but also provided a means of measuring its strength. The apparatus is described in more detail in Bruce, 1970.

Chemicals

Caffeine and theophylline, obtained from Sigma Chemical Co., were each added to final concentrations of 3, 5, or 10 mM to two replicate samples of each mating type.

Results

The period lengthening of the phototactic rhythm of mt$^-$ *Chlamydomonas* by caffeine treatment is shown in Figure 1. The effect is already apparent in the second peak of the rhythm, even at the lowest concentration tested (3 mM). When treated with 10 mM caffeine, the cultures became arrhythmic after 2–3 days.

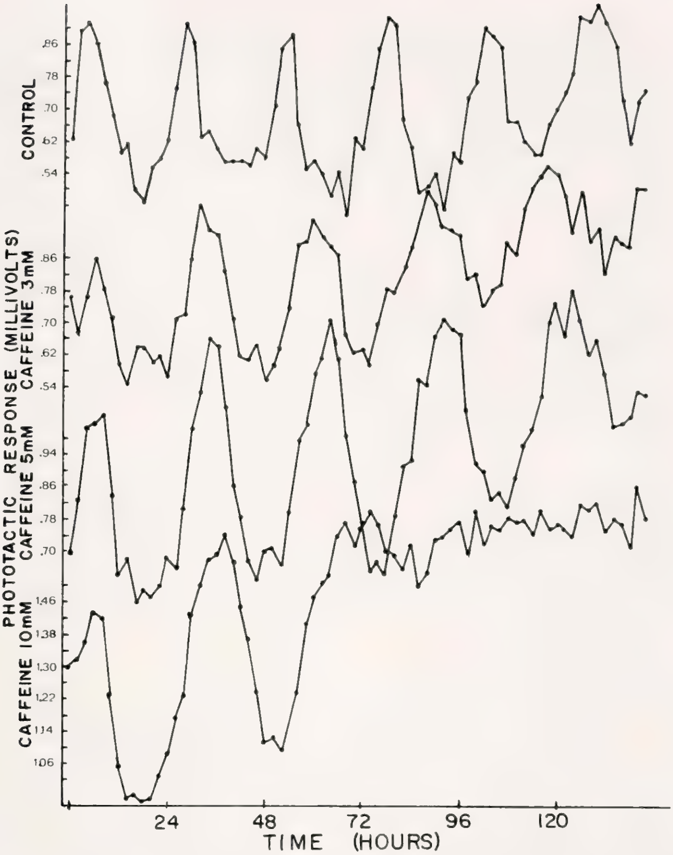


FIGURE 1. The rhythmic phototactic response of the − mating type of strain **137 F** of *Chlamydomonas reinhardii* and its modification by treatment with caffeine. Each point represents the mean of two samples.

Figure 2 shows the period lengthening of the phototactic rhythm of mt$^-$ caused by theophylline treatment. Although the period increases are not as great as those caused by equal concentrations of caffeine, a slight lengthening is evident by the second day of treatment. The data suggest that the phototactic response becomes arrhythmic after about 5 days of exposure to 5 mM theophylline. But the rhythm was only followed for 6 days, so it is uncertain whether the 6th peak would have been present.

A comparison of the period lengthening effects of caffeine and theophylline on both mt$^+$ and mt$^-$ *Chlamydomonas* is presented in Figure 3. The caffeine increased the period length more than theophylline did and the responses of mt$^+$ and mt$^-$ were very similar, although the period increase of mt$^-$ was slightly greater at almost all drug concentrations. The period lengthening with both drugs was dose-related. A 3 mM caffeine treatment caused a 9.6% increase in the rhythm's period length in mt$^+$ and a 12.7% increase in that in mt$^-$. With 5 mM caffeine, the period lengths increased 12.9% in mt$^+$ and 16.9% in mt$^-$. The increases in the period lengths were substantially greater with a 10 mM caffeine treatment: 27 and 31% in mt$^+$ and mt$^-$, respectively. The theophylline treatments generated

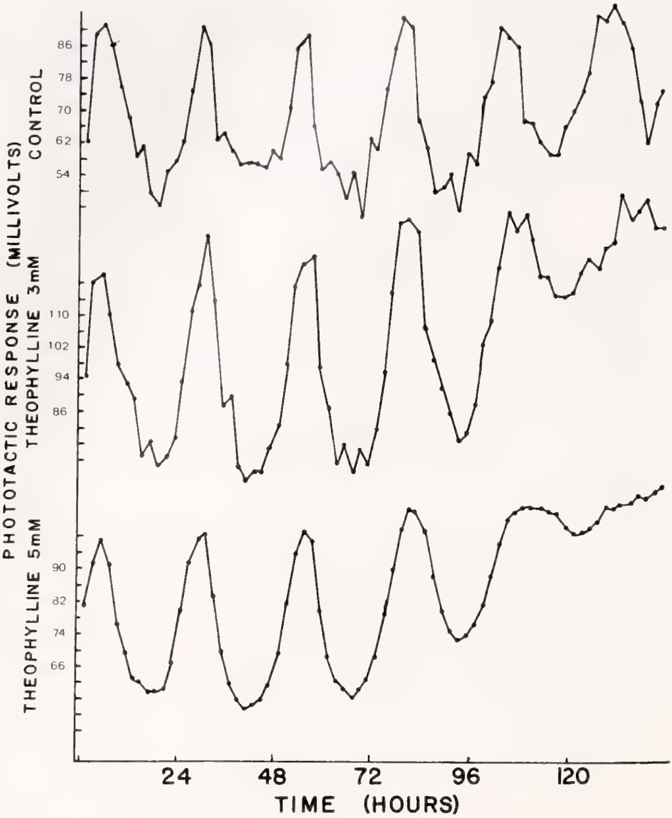


FIGURE 2. The rhythmic phototactic response of the − mating type of strain 137 F of *Chlamydomonas reinhardii* and its modification by treatment with theophylline. Each point represents the mean of two samples.

smaller changes in period length. At 3 mM there was a 5.5% increase in mt^+ and 2.8% increase in mt^-. Five mM theophylline resulted in 5.7 and 6.4% increases in the period lengths of mt^+ and mt^-, respectively.

DISCUSSION

Theophylline and caffeine both increased the period length of the phototactic rhythm of *Chlamydomonas* almost immediately and in a dose-related manner. These results are consistent with previous tests of the effects of these substances, but extend the findings to a new circadian system. In previous studies it was shown that sustained administration of theophylline produced an increase in the period length of the sleep movement rhythm of the bean plant, *Phaseolus* (Keller, 1960), of the conidiation rhythm of the bread mold *Neurospora* (Feldman, 1975) and of the leaf movement rhythm of *Trifolium* (Bollig *et al.*, 1978). Pulses of theophylline produced phase changes in the sleep movement rhythm of *Phaseolus* (Mayer *et al.*, 1975) and in the body temperature rhythm of *Rattus rattus* (Ehret *et al.*, 1975). Caffeine has been less widely tested. When continuously administered, it caused period lengthening in *Neurospora*'s conidiation rhythm

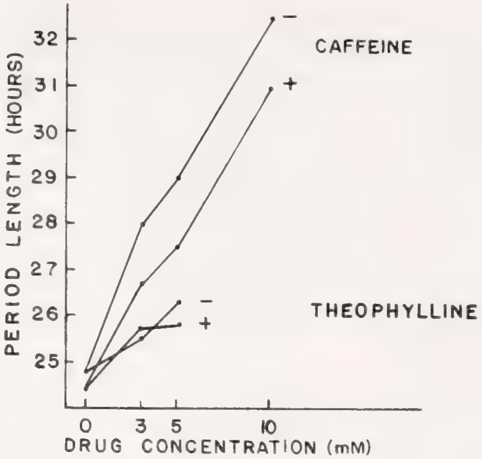


FIGURE 3. Period lengthening effects of caffeine and theophylline on both the + and − mating types of strain 137 F of *Chlamydomonas reinhardii*. Each point represents the mean of two replicate samples. For 9 of the 12 points the standard deviations ranged from 0 to 0.35. For the + mating type 5 mM and the 10 mM caffeine-treated samples, the standard deviations were 0.71 and 1.4, respectively. For the − mating type the standard deviation for treatment with 10 mM caffeine was 0.71.

(Feldman, 1975), and when pulsed, it produced phase changes in the *Phaseolus* sleep movement rhythm (Mayer and Scherer, 1975).

In our experiments caffeine was found to have a much greater effect on the biological timing process than did theophylline at equal concentrations. This is consistent with the results of Feldman (1975) on the conidiation rhythm of *Neurospora*.

The period lengthening effect of these drugs suggests that they are acting at or close to the biological timing process. The best known effect of these drugs is the inhibition of cAMP phosphodiesterase, which results in an increase in cellular cAMP levels. Amrhein and Filner (1973) demonstrated that theophylline inhibits cyclic AMP phosphodiesterase in *Chlamydomonas*.

However, other experiments suggest that the drugs may not be influencing the clock through their alteration of cAMP levels. Scott and Solomon (1973) found that in *Neurospora*, theophylline (5 mM) inhibited phosphodiesterase more effectively than caffeine; 83% of the phosphodiesterase activity remained in the caffeine-treated samples and only 52% of the activity remained in the theophylline-treated samples. If the action of the drugs on the timing mechanism of the clock is via their effects on cAMP phosphodiesterase, it is surprising that in Feldman's (1975) study on the conidiation rhythm of *Neurospora* and in this experiment, caffeine was found to increase the period length considerably more than theophylline. In addition, Feldman *et al.* (1979) have shown that mutants of *Neurospora* that have less than 1% of the normal levels of cyclic AMP still have a normal clock, indicating that the major pools of cAMP in the cell are not essential for normal clock operation.

Furthermore, if theophylline affected the clock by altering cAMP levels through its effect on phosphodiesterase, one might expect that pulses of cAMP and of theophylline would have similar effects on the clock and that these would be observed as similar phase-response curves. However, although both cAMP and theophylline increase the period length of the *Trifolium* leaf movement rhythm, when the drugs were administered as pulses the phase-response curve for cAMP had only delays, while the one for theophylline had both advances and delays (Bollig *et al.,* 1978).

Another way to alter cAMP metabolism would be to inhibit adenyl cyclase activity with a substance such as quinidine. It has been demonstrated that quinidine (26–58 μg/ml) has no effect on the phototactic rhythm of *Chlamydomonas* (Carter, 1975), although higher concentrations (259 μg/ml) inhibited phototaxis completely. If caffeine and theophylline were slowing biological timing processes through their effects on cAMP levels, one would expect that quinidine would also affect the system.

Caffeine and theophylline are known to have effects on other cellular processes, such as ion transport, macromolecular synthesis and the phosphorylation of proteins. Perhaps it is better to consider the period lengthening effects of these drugs as consistent with hypotheses that involve these cellular processes.

LITERATURE CITED

AMRHEIN, N., AND P. FILNER, 1973. Adenosine 3′ : 5′ cyclic monophosphate in *Chlamydomonas reinhardtii:* isolation and characterization. *Proc. Nat. Acad. Sci. U.S.A.,* **70**: 1099–1113.

BOLLIG, I., K. MAYER, W-E. MAYER, AND W. ENGELMANN, 1978. Effects of cAMP, theophylline, imidazole, and 4-(3,4-dimethoxybenzyl)-2-imidazolidone on the leaf movement rhythm of *Trifolium repens*—a test of the cAMP hypothesis of circadian rhythms. *Planta,* **141**: 225–230.

BRINKMANN, K., 1971. Metabolic control of temperature compensation in the circadian rhythm of *Euglena gracilis.* Pp. 567–593 in M. Menaker, Ed., *Biochronometry.* National Academy of Sciences, Washington, D. C.

BRINKMANN, K., 1973. The role of actidion in the temperature jump response of the circadian rhythm in *Euglena gracilis.* Pp. 523–529 in B. Chance, Ed., *Biological and Biochemical Oscillators.* Academic Press, New York.

BRINKMANN, K., 1974. The effect of ethanol on the metabolism and circadian rhythms of *Euglena gracilis. J. Interdiscip. Cycle Res.,* **5**: 186.

BRINKMANN, K., 1976. The influence of alcohols on the circadian rhythm and metabolism of *Euglena gracilis. J. Interdiscip. Cycle Res.,* **7**: 149–170.

BRUCE, V. G., 1970. The biological clock in *Chlamydomonas reinhardi. J. Protozool.,* **17**: 328–334.

BRUCE, V. G., 1972. Mutants of the biological clock in *Chlamydomonas reinhardi. Genetics,* **70**: 536–548.

BÜNNING, E., AND J. BALTES, 1962. Wirkung von Äthylalkohol auf die physiologische Uhr. *Naturwissenschaften,* **49**: 19–20.

BÜNNING, E., AND I. MOSER, 1973. Light-induced phase shifts of the circadian leaf movements of *Phaseolus:* comparison with the effects of potassium and ethyl alcohol. *Proc. Nat. Acad. Sci. U.S.A.,* **70**: 3387–3389.

CARTER, A., 1975. Antibiotic effects on the phototactic response of *Chlamydomonas reinhardi.* Sr. Honors thesis, Princeton University.

CUMMINGS, F. W., 1975. A biochemical model of the circadian clock *J. Theoret. Biol.,* **55**: 355–470.

EHRET, C. F., N. R. POTTER, AND K. W. DOBRA, 1975. Chronotypic action of theophylline and of pentobarbital as circadian zeitgeibers in the rat. *Science,* **198**: 1212–1215.

ENGELMANN, W., 1972. Lithium slows down the *Kalanchoe* clock. *Z. Naturforsch.,* **27b**: 477.

ENGELMANN, W., 1973. A slowing down of circadian rhythms by lithium ions. *Z. Naturforsch.,* **28c**: 733–736.

ENGELMANN, W., A. MAUER, M. MUHLBACH, AND M. JOHNSON, 1974. Action of lithium ions and heavy water in slowing circadian rhythms in petal movement in Kalanchoe. *J. Interdiscip. Cycle Res.,* **5**: 199–205.

ENRIGHT, J. T., 1971. The internal clock of drunken isopods, *Z. Vgl. Physiol.*, **72** : 1–16.

ESKIN, A., 1972. Phase shifting a circadian rhythm in the eye of *Aplysia* by high potassium pulses. *J. Comp. Physiol.*, **80** : 353–376.

ESKIN, A., 1977. Neurophysiological mechanisms involved in the photoentrainment of the circadian rhythm from the *Aplysia* eye. *J. Neurobiol.*, **8** : 273–299.

FELDMAN, J., 1967. Lengthening the period of a biological clock in *Euglena* by cycloheximide an inhibitor of protein synthesis. *Proc. Nat. Acad. Sci. U.S.A.*, **57** : 1080–1087.

FELDMAN, J., 1975. Circadian periodicity in *Neurospora:* alteration by inhibitors of cyclic AMP phosphodiesterase. *Science*, **190** : 789–790.

FELDMAN, J. F., G. GARDNER, AND R. DENISON, 1979. Genetic analysis of the circadian clock of *Neurospora*. Pp. 57–66 in M. Suda, O. Hayaishi and H. Nagagawa, Eds., *Biological rhythms and their central mechanism,* Elsevier/North Holland Biomedical Press.

HASTINGS, J. W., 1960. Biochemical aspects of rhythms : phase shifting by chemicals. *Cold Spring Harbor Symp. Quant. Biol.*, **25** : 131–143.

JACKLET, J. W., 1977. Neuronal circadian rhythm : phase shifting by a protein synthesis inhibitor. *Science*, **198** : 69–71.

KARAKASHIAN, M. W., AND J. W. HASTINGS, 1962. The inhibition of a biological clock by actinomycin D. *Proc. Nat. Acad. Sci. U.S.A.*, **48** : 2130–2137.

KARAKASHIAN, M. W., AND J. W. HASTINGS, 1963. The effects of inhibitors of macromolecular biosynthesis upon the persistent rhythm in luminescence in *Gonyaulax*. *J. Gen. Physiol.*, **47** : 1–12.

KARAKASHIAN, M. W., AND H-G. SCHWEIGER, 1976a. Evidence for a cycloheximide-sensitive component in the biological clock of *Acetabularia*. *Exp. Cell Res.*, **98** : 303–312.

KARAKASHIAN, M. W., AND H-G. SCHWEIGER, 1976b. Temperature dependence of the cycloheximide-sensitive phase of the circadian cycle in *Acetabularia mediterranea*. *Proc. Nat. Acad. Sci. U.S.A.*, **73** : 3216–3219.

KELLER, S., 1960. Uber die Wirkung chemischer Faktoren auf die tagesperiodischen Blattbewegungen von *Phaseolus multifloris*. *Z. Bot.*, **48** : 32–57.

MAYER, W., R. GRUNER, AND H. STRUBEL, 1975. Periodenverlangerund der circadianen Rhythmik von *Phaseolus coccineus* durch theophyllin. *Planta*, **125** : 141–148.

MAYER, W., AND I. SCHERER, 1975. Phase shifting effect of caffeine in the circadian rhythm of *Phaseolus coccineus*. *Z. Naturforsch*, **30C** : 855–856.

MERGENHAGEN, D., AND H-G. SCHWEIGER, 1975a. Circadian rhythm of oxygen evolution in cell fragments of *Acetabularia mediterranea*. *Exp. Cell Res.*, **92** : 127–130.

MERGENHAGEN, D., AND H-G. SCHWEIGER, 1975b. The effect of different inhibitors of transcription and translation on the expression and control of circadian rhythms in individual cells of *Acetabularia*. *Exp. Cell. Res.*, **94** : 321–326.

ROTHMAN, B. S., AND F. STRUMWASSER, 1977. Manipulations of a neuronal oscillator with inhibitors of macromolecular synthesis. *Fed. Proc.* **36** : 2050–2066.

SCOTT, W. A., AND B. SOLOMON, 1973. Cyclic 3′,5′-AMP phosphodiesterase of *Neurospora crassa*. *Biochem. Biophys. Res. Commun.*, **53** : 1024–1030.

SWEENEY, B. M., 1974. The potassium content of *Gonyaulax polyedra* and changes in the circadian rhythm of stimulated bioluminescence by short exposure to ethanol and valinomycin. *Plant Physiol.*, **53** : 337–342.

SWEENEY, B. M., 1976. Evidence that membranes are components of circadian oscillators. Pp. 267–282 in J. W. Hastings and H-G. Schwieger, Eds. *The Molecular Basis of Circadian Rhythms,* Abakon Verlagsgestellschaft, Berlin.

SWEENEY, B. M., C. F. TUFFLI, AND R. H. RUBIN, 1967. The circadian rhythm in photosynthesis in *Acetabularia* in the presence of actinomycin D, puromycin and chloramphenicol. *J. Gen. Physiol.*, **50** : 647–659.

WALZ, B., AND B. M. SWEENEY, 1979. Kinetics of the Cycloheximide-induced phase changes in the biological clock in *Gonyaulax*. *Proc. Nat. Acad. Sci. U.S.A.*, **76** : 6443–6447.

Reference: *Biol. Bull.*, **159**: 656–668. (December, 1980)

COMPARATIVE STUDY OF THE BLOOD PLASMA OF THE ASCIDIANS *PYURA STOLONIFERA* AND *ASCIDIA CERATODES*

CLIFFORD J. HAWKINS,[1] PAULINE M. MEREFIELD,[1] DAVID L. PARRY,[1]
WILTON R. BIGGS,[2] AND JAMES H. SWINEHART [2]

[1] *Department of Chemistry, University of Queensland, Brisbane, Australia, 4067; and*
[2] *Department of Chemistry, University of California, Davis, California 95616*

ABSTRACT

The components of the blood plasma of the ascidians, *Pyura stolonifera* and *Ascidia ceratodes*, have been separated by Sephadex G75 chromatography and gel electrophoresis. The absorption spectra of the plasma and the fractions for both species and the corresponding circular dichroism spectra for *Pyura stolonifera* have been recorded. The components for each species consisted of a number of proteins and two N-acetylaminosugar compounds, probably aminopolysaccharides. Although there were similarities in the properties of these components between the two species, a number of important differences were observed, especially with regard to the uptake of metals as studied by ^{59}Fe, ^{54}Mn, ^{48}V, and ^{51}Cr radiotracers. Electron spin resonance measurements detected manganese(II) in a N-acetylaminosugar component of the plasma of *Pyura stolonifera*.

INTRODUCTION

The blood of each suborder of the Ascidiacea (Aplousobranchia, Phlebobranchia, and Stolidobranchia) seems to offer its own unusual characteristics. For example, members of the Phlebobranchia extract vanadium from sea water and concentrate it in morula blood cells called vanadocytes. Vanadium is also observed in some species within the Aplousobranchia, but where it is found within the animal is unknown (Biggs and Swinehart, 1976). Ascidians containing only iron as the dominant biological metal are found in the suborders Aplousobranchia and Stolidobranchia. For instance, dry cells of the stolidobranch *Pyura stolonifera* contain up to 5.1 mg/g iron, localized in blood cells termed ferrocytes (Endean, 1955a). Iron is also observed in the blood cells of vanadium-containing ascidians, but at an order of magnitude less than the vanadium. For example, *Ascidia ceratodes* blood cells contain 0.5 mg/g dry weight iron and 6–8 mg/g vanadium (Swinehart *et al.*, 1974).

Besides vanadium and iron, a number of other metals have been detected in ascidians. Manganese has been found in *Ciona intestinalis* (Noddack and Noddack, 1940; Carlisle, 1968), *Didemnum albidium* (Noddack and Noddack, 1940), *Didemnum candidum* (Carlisle, 1968), and *Pyura stolonifera* (D. A. Buckingham and J. M. Webb, Australian National University, personal communication). Chromium has been detected in the body tissue of *Ciona intestinalis* (Bielig *et al.*, 1961), and in whole zooids (Levine, 1962) and whole colonies (Swinehart *et al.*, 1974) of *Eudistoma ritteri*. It can be argued that these observations represent localized

Received July 20, 1979; accepted August 20, 1980.

Abbreviations: 2,4,6-tripyridyl-s-triazine (TPTZ), periodic acid Shiff (PAS), circular dichroism (CD), counts per minute (CPM).

metal concentrations in sea water reflected in analyses of total animals. It is important to learn whether ascidians take up metals such as manganese and chromium and incorporate them in their plasma and blood cells.

In any process in which the final repository of the metal is blood cells, it would seem logical that the metal would make a transient or permanent appearance in plasma. To this end, the properties of the plasma of two phylogenetically diverse solitary ascidians, *Pyura stolonifera* and *Ascidia ceratodes*, have been examined to ascertain the similarities and differences between them. *P. stolonifera* is an iron-containing species in the suborder Stolidobranchia, and *A. ceratodes* is a vanadium-containing species in the suborder Phlebobranchia. The interaction of the plasma with metal ions has been examined by studying the uptake of ^{59}Fe (as Fe(III)), ^{54}Mn (as Mn(II)), ^{51}Cr (as Cr(III)), and ^{48}V (as V(V)) by animals and plasma.

MATERIALS AND METHODS

Specimens of the solitary ascidians *A. ceratodes* and *P. stolonifera* were collected at Bodega Bay (California), U.S.A.; and Hastings Point (New South Wales), Stradbroke Island, and Noosa (Queensland), Australia, respectively. Both species were maintained in aquaria in aerated local sea water. Specimens of *A. ceratodes* showed changes in properties of the plasma after short-term residence in an aquarium, so they were kept a minimum time under such conditions. The specimens of *P. stolonifera* studied could be kept in aquaria for long periods, even in excess of 6 months, without significant change in blood composition provided water temperature was maintained between 15° and 19°C and the aquaria had a balanced ecology. If the water temperature exceeded 19°C, for example in summer with no cooling, the blood cell count sank to a very low level in healthy animals and mortality increased markedly.

Blood samples were removed from *A. ceratodes* by cardiac puncture, and from *P. stolonifera* by cutting the base and creating a small depression into which blood flowed. Blood cells were removed from the plasma by centrifugation. The plasma of both species becomes cloudy if left standing for a short time, so samples were used soon after blood was removed from the animal and centrifuged. The nature of the white cloudy material is unknown.

The plasma samples were tested for N-acetylaminosugars (Reissig *et al.*, 1955), protein (biuret test), and after digestion with $HClO_4$-HNO_3 mixture (Allan, 1959), for iron (colorimetrically using 2,4,6-tripyridyl-s-triazine [TPTZ] [Collins *et al.*, 1959], and by atomic absorption).

Gel electrophoretic experiments were carried out using analytical (6 or 8% polyacrylamide) and, in some cases, stacking gels prepared by standard techniques. Bromophenol blue was used as the tracking dye, and the current was 2–3 ma/gel. The gels were fixed with 15% trichloroacetic acid, stained with 1% Coomassie Brilliant Blue, and destained with 5% glacial acetic acid for protein analysis; for carbohydrate analysis the gels were treated with aqueous 40% ethanol and 5% glacial acetic acid, then oxidized with periodic acid, and finally stained with Schiff base stain (PAS) (Pearse, 1960).

In the *in situ* radioactivity experiments about 4–15 liters of sea water was inoculated with an appropriate isotope, ^{59}Fe, ^{54}Mn, ^{48}V, or ^{51}Cr, and allowed to equilibrate for several hours. Animals were added and air was bubbled through the system. Animals were removed at various times and their blood removed and analyzed as described above. Plasma was either separated using liquid chro-

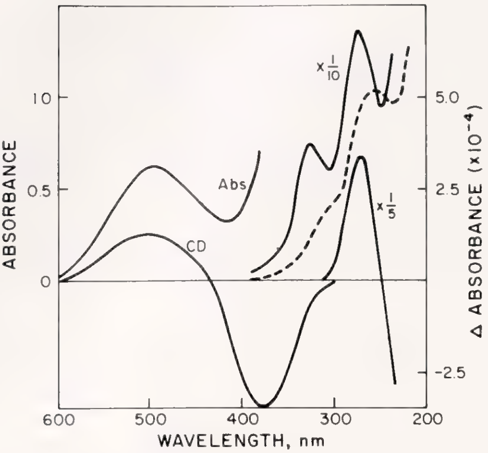


FIGURE 1. Ultraviolet visible and circular dichroism (CD) spectra of the fresh plasma from *P. stolonifera* (solid line) and *A. ceratodes* (dashed line). Cell pathlength 1 cm. Dilution shown where applicable.

matography (Sephadex) or gel electrophoresis and counted, or counted directly. In plasma inoculation experiments, plasma was maintained at 4°C for *P. stolonifera* and at 12°C for *A. ceratodes*. For both types of experiments with *P. stolonifera*, the plasma was dialyzed against distilled water for 1 hr before counting or chromatography, and the individual chromatography fractions were dialyzed for a further 2 hr before counting. In some cases solid samples (*e.g.*, test, hydroids, algae) were counted, and where possible the counts were reduced to a per minute per gram basis. The counting instruments used were a Beckman Gamma 310 Radiation Counter (*A. ceratodes*) and a Nuclear Chicago counting well system (*P. stolonifera*).

Spectroscopic studies employed Cary Models 17 and 14 spectrophotometers for the ultraviolet (UV) visible spectra, a Jobin Yvon Mark III Dichrograph for the circular dichroism (CD) spectra, a Varian V 4502 15X band spectrometer operating at 9.104 GHz for the ESR spectra, a Varian Techtron Model 1200 spectrometer for the atomic absorption studies, and a Lab Test Multielement Analyser for the inductively coupled plasma studies. For ESR measurements, fractions of the plasma were separated by chromatography, dialyzed against distilled water for 3 hr at 10°C, freeze dried, and studied as powders at 77°K.

RESULTS

Properties of the plasma of the iron-containing ascidian *P. stolonifera* and the vanadium-containing ascidian *A. ceratodes* are presented in two parts: spectral and separative properties of the plasma and its constituents, and association of metals with the plasma or its constituents. Association was measured by direct analysis, electron spin resonance (ESR), or radiotracer incorporation.

Spectral and separative properties

Plasma from *P. stolonifera* is pink; that of *A. ceratodes* is yellow. Figure 1 contains the UV visible spectra of both species. Common to both spectra were bands in the 260–275 and 300–330 nm regions. In addition, *P. stolonifera* had an absorbance at about 500 nm which imparted the pink color to its plasma. The CD spectrum of the plasma of *P. stolonifera* is recorded in Figure 1 for comparison with chromatographed constituents of the plasma.

The plasmas of both species were chromatographed through Sephadex G75 with 0.05M NaCl as eluent. The elution patterns detected at 275 nm for both species and also at 325 nm for *P. stolonifera* are given in Figure 2. For both species two major bands, one each, in fractions 1–2 and 8–13 were observed. The second band from *P. stolonifera* could contain several components. In addition, a pink compound in fractions 3–5 was observed between the two major bands. The first band eluted (fractions 1–2) was in the void volume of the column and should have contained compounds with molecular weights greater than the exclusion of the G75 column (30,000). It gave a positive test for protein, and negative tests for N-acetylaminosugar and iron for both species. It had a maximum at 275 nm in the absorption spectrum for *P. stolonifera* and at 282 for *A. ceratodes.*

Fractions 3–5 differed for the two species. Both give positive tests for protein and negative tests for N-acetylaminosugar and iron. However, the absorption spectrum for *P. stolonifera* (Fig. 3) contained maxima at 497 and 275 nm, whereas the 497 nm band was missing for *A. ceratodes.* This pink band from *P. stolonifera* was eluted with water in fractions 2–4. The CD spectrum of the pink band had a positive Cotton effect at 516 nm, a negative at 387 nm, and a positive at 293 nm. Below 260 nm the CD was negative.

Fractions 8–13 for *A. ceratodes* had absorption maxima at 260 and 314 nm with the ratio of the absorbance at the two wavelengths increasing as the fraction number increases, suggesting that more than one compound was present. For *P. stolonifera,* on some occasions only a 327-nm maximum was observed in fractions 8–13, and on these occasions the spectrum did not change significantly across the band. On other occasions a 275-nm maximum was obtained, and the absorbance ratio for 275 and 325 nm increased with increasing fraction number, again showing the presence of more than one compound. When fractions from *P. stolonifera* were dialyzed, a compound having the UV visible and CD spectra shown in Figure 3 was isolated in the external solution. The same dialysis properties were observed for comparable fractions from *A. ceratodes,* and resulted in the separation of a

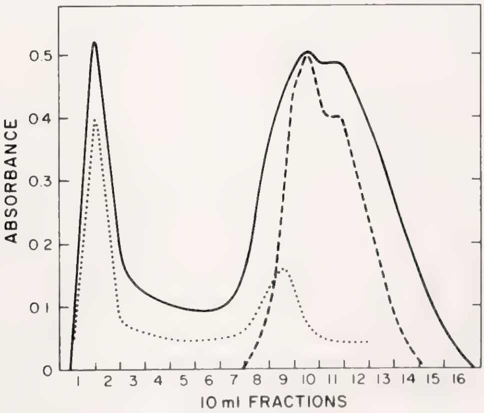


FIGURE 2. Elution pattern of plasma of *P. stolonifera* from Sephadex G75 at 275 (solid line) and 325 (dashed line) nm, and *A. ceratodes* at 275 nm (dotted line). Fractions 3–5 from *P. stolonifera* were pink.

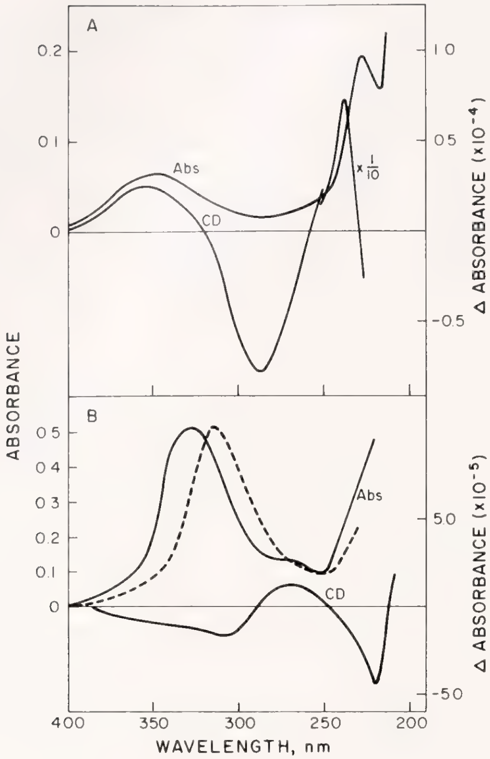


FIGURE 3. Absorption and CD spectra of (A) the "pink fraction" (fractions 3–5) from the plasma of *P. stolonifera,* and (B) the "327 nm" compound isolated from the plasma of *P. stolonifera* (solid line) and the absorption spectrum of the "314 nm" compound from *A. ceratodes* (fractions 8–13) (dashed line).

compound having a 314 nm maximum (Fig. 3). The separation in this case was not as clean.

An alternate method for separating the *P. stolonifera* compounds with 327 and 275 nm absorption was to freeze dry the fractions when water was used as eluent. The aqueous extract of the residue gave the same spectrum as in Figure 3A. A white insoluble residue became purple with time.

Fractions 8–13 and isolated compounds from both species tested positive for N-acetylaminosugar, and negative for protein. With 0.05 M NaCl eluent, the *P. stolonifera* fractions tested positively for iron. But this appeared to be due to a contaminant in the sodium chloride, because when distilled water was used as eluent, a negative test for iron was obtained. *A. ceratodes* tested negative for iron.

Both species gave different elution patterns if subjected to trauma during transportation or in aquaria. When species of *A. ceratodes* were transported long distances and/or remained in an aquarium for as little as 2 days, the pattern had increased absorption for fractions 3–7, resulting from increasing absorption below 300 nm, showing no maximum down to 200 nm. *A. ceratodes* seemed more sensitive to these influences in winter. *P. stolonifera* appeared to be more robust, but if subjected to trauma due to temperature increase, for example in summer, all fractions except 1 and 2 showed markedly decreased absorption.

The gel electrophoretic patterns (Fig. 4) for plasma showed a saccharide band (stained by PAS) at R_fO for both species and a number of protein bands (stained by Coomassie Blue). For *P. stolonifera,* six protein bands were observed with the major component at R_f 0.45. Two patterns (A and B in Fig. 4) were observed for the protein bands in the *A. ceratodes* plasma from animals collected from two sites in Bodega Bay, California. The pattern was not related to the collection site. Gel electrophoresis of fraction 2 from *P. stolonifera* showed a major and three minor protein bands, whereas for *A. ceratodes* two protein bands were observed. The gel electrophoresis pattern of fraction 5 (*P. stolonifera*) usually contained two major bands that were stained by Coomassie Blue, but for some animals four separate protein bands were observed. For *A. ceratodes* at least five proteins were present in this fraction. Fractions 8–13 for both species yielded no separation of the different compounds on the gel electrophoretogram, with only a band (positive for saccharide) observed at R_fO.

Metal association studies

One reason for interest in the elution and gel electrophoretic patterns is to learn which plasma constituents, if any, participate in the uptake of metals and their subsequent transport to the blood cells. Therefore, uptake studies involving Fe, Mn, Cr, and V using the radiotracers ^{59}Fe, ^{54}Mn, ^{51}Cr, and ^{48}V were carried out. In addition, cells and animal surface (test) were analyzed.

Animals of both species were incubated with ^{59}Fe for up to 150 hr. Plasma activity levels of both species increased with time, whereas the sea water activity decreased rapidly to approximately a fifth of its original value at 150 hr. The Fe level increased in the blood cells as well as in the plasma. On a counts per minute (CPM) per ml whole-blood basis, the ^{59}Fe content of the blood cells exceeded that

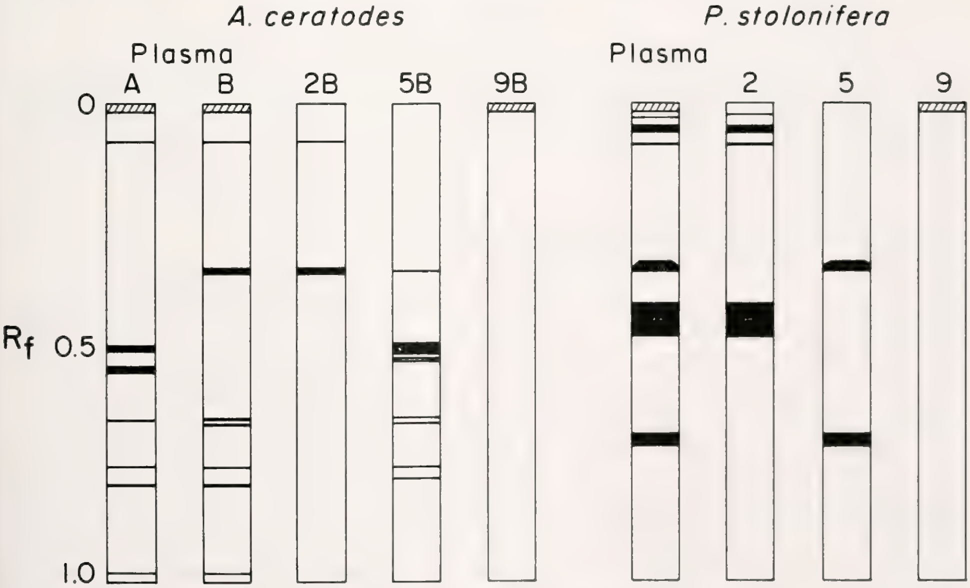


FIGURE 4. Gel electrophoretograms: 8 and 6% polyacrylamide gels, respectively, were used for plasma and Sephadex separated fractions of *A. ceratodes* and *P. stolonifera.* Bands stained by Coomassie Blue are shown in solid lines and those stained by PAS in broken lines.

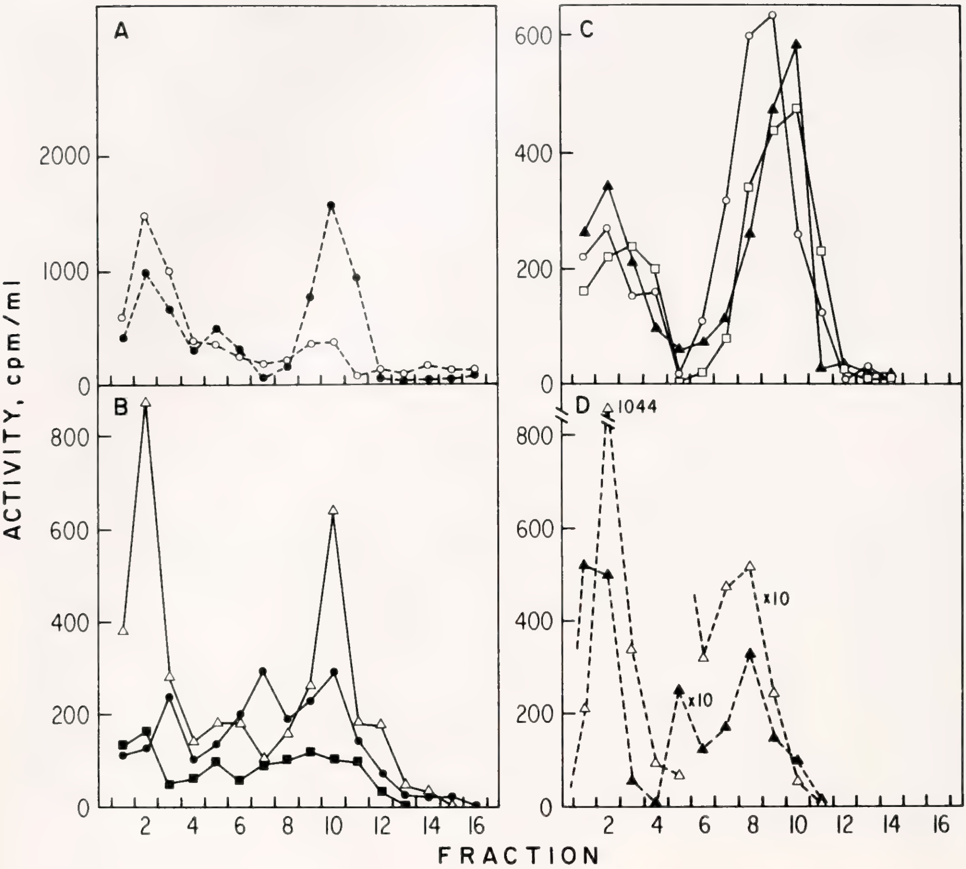


FIGURE 5. Elution patterns for ^{59}Fe activity: In plasma of *P. stolonifera* for two sets of whole animal experiments (A) 48 hr (closed circles), 118 hr (open circles), and (B) 24 hr (closed circles), 72 hr (closed squares) and 120 hr (open triangles); (C) in plasma incubation of *P. stolonifera*: 0.5 hr (open circles), 24 hr (open squares), 60 hr (closed triangles; (D) in whole animal incubation of *A. ceratodes*: 48 hr (closed triangles), 144 hr (open triangles).

of the plasma at any time for either species. Upon lysing the blood cells, ^{59}Fe activity appeared in both the lysate and residue.

Iron 59 activity versus plasma fraction eluted from Sephadex G75 for both species is summarized in Figure 5. In the *P. stolonifera* whole-animal incubation experiments (Figs. 5A, B), ^{59}Fe activity correlated well with the three elution bands discussed earlier: fractions 1–2, 3–5, and 8–13. Even when the 275–497 nm compound normally associated with fractions 3–5 was shifted to fractions 5–7 in the 24 hr sample (Fig. 5B), the ^{59}Fe activity also shifted. When *P. stolonifera* plasma was incubated, a different pattern (C in Fig. 5) was obtained. The activity was not maximized where the 275–497 nm compound was eluted (normally fractions 3–5). Indeed at fraction 5, the activity was at a minimum.

For *P. stolonifera,* the pattern of ^{59}Fe uptake was independent of iron concentration. This was not the case for *A. ceratodes.* At low activity levels (0.1 μCi in 4 1 sea water) activity was restricted to fractions 8–13, whereas at higher Fe levels some activity was found in all peaks eluted from Sephadex G75, with similar patterns obtained from whole animal and plasma incubations (D in Fig. 5).

TABLE I

^{59}Fe radiotracer results for organs and sections of test of Pyura stolonifera. *Test sections had the dimensions 0.5 × 1.5 × 1.0 cm and were consecutive slices taken from a cross-section of the base with T_1 adjacent to the exterior.*

Organ or test section	Radioactivity (CPM/g)		
	24 hr incubation	72 hr incubation	120 hr incubation
Gut	14,730	32,950	2,300
Sac	2,250	7,530	2,550
Gonad	160	180	82
Liver	2,547	19,700	11,580
T_1	780	690	330
T_2	560	2,600	762
T_3	2,970	630	630

When sea water inoculated with ^{59}Fe was chromatographed through Sephadex G75 using 0.05 M NaCl as eluent, the free metal was eluted in fractions 8–13, the same fractions as the "314 nm" and "327 nm" compounds from *A. ceratodes* and *P. stolonifera,* respectively. To test whether the ^{59}Fe detected in fractions 8–13 in the above experiments was free or bound, the plasma fractions were dialyzed for 3 hr before counting. The activity remained, showing the ^{59}Fe was bound to the organic compound(s).

Although the various fractions took up ^{59}Fe in the whole animal and plasma experiments with both species, no natural iron was detected by the TPTZ method or by atomic absorption for these fractions. However, an inductively coupled plasma study of the isolated "327 nm" compound and the insoluble residue from fraction 8–15 of *P. stolonifera* found that the "327 nm" compound had 0.05% iron whereas the insoluble fraction had 0.74% iron. We concluded that the natural concentrations of iron in these fractions were too low to be detected by the TPTZ or atomic absorption methods, given the small amounts of materials used.

When ^{59}Fe was administered to *A. ceratodes* and *P. stolonifera,* significant activity appeared on the surface (test) of the animals. This means that much of the ^{59}Fe was unavailable for uptake by the internal organs and raises the question of the reason for surface absorption. In a typical experiment using *A. ceratodes,* comparable 1 cm^2 areas of the outer surface of the test had 28,300 CPM (48 hr) and 25,000 CPM (144 hr) while the same area 1 mm below the test had 50 CPM (48 hr) and 800 CPM (144 hr). Since blood vessels run through the test, some of the activity observed below the surface of the test could be due to incorporation of ^{59}Fe into the plasma. Table I shows the incorporation of ^{59}Fe into various *P. stolonifera* organs and layers of test. Again, the surface of the test accumulated ^{59}Fe and part of the activity below the surface must have arisen from accumulation of Fe in the blood. For both *A. ceratodes* and *P. stolonifera* the chemical composition of the test guaranteed that it would be a good complexing agent for metals, and Fe accumulation on the surface would be expected. Endean examined the test of *P. stolonifera* and found it to be composed of polysaccharides and aminopolysaccharides (Endean, 1955b).

An interesting sidelight on ^{59}Fe incorporation is its uptake by algae, hydroids, and possibly bacteria attached to the surface of *P. stolonifera* and *A. ceratodes.* Table II shows CPM/g dry weight for algae and hydroids found on the surface of

TABLE II

^{59}Fe uptake by algae and hydroid on surface of Pyura stolonifera

Organism	Radioactivity (CPM/g)		
	24 hr incubation	72 hr incubation	120 hr incubation
Gracilaria edulis (Rhodophyte)	31,960	36,550	11,710
Halimeda discoidea (Chlorophyte)	—	39,450	9,450
Caularpa nexicana (Chlorophyte)	—	81,330	25,120
Hydroid sp.	123,000	26,860	127,000

P. stolonifera. Comparable observations were made for algae associated with *A. ceratodes.* Manganese 54 uptake experiments with *A. ceratodes* showed considerable uptake by a hydroid attached to the surface. Clearly some of the ^{59}Fe and ^{54}Mn on the test of the two species resulted from uptake by organisms on the surface.

Whole-animal ^{54}Mn studies for both species showed uptake in the plasma and blood cells. In both animal and plasma incubation experiments the activity of ^{54}Mn in the plasma was maximized at the same position in the elution pattern from Sephadex G75 as the N-acetylaminosugar (fractions 8–13). When sea water was inoculated with ^{54}Mn as $Mn(OH_2)_6^{2+}$ and chromatographed, it too gave a peak at this position. However, ^{54}Mn in the plasma fractions was not removed by short term dialysis. During whole-animal incubation experiments the sea water activity decreased relatively rapidly with time, reaching a tenth of its original activity in less than 100 hr.

For *P. stolonifera*, the "327 nm" compound and its associated insoluble material from fractions 8–13 were analyzed for manganese by inductively-coupled plasma optical emission. Values of 0.04 and 0.12%, respectively, were obtained. ESR spectra of the freeze-dried chromatographic fractions (distilled water as eluent) of *P. stolonifera* gave no signal for fractions 1–7 but gave an intense typical six-line Mn(II) resonance (see, for example, Chan *et al.*, 1967; Meirovitch and Lanir, 1978) with $g = 2.001 \pm 0.001$ and $A = 92$ G for the "330" band. The quantities

TABLE III

Absorbance ratio for "275" and "330" bands, and the positions of the maxima (in nm, in parenthesis) for fractions 9–12 from chromatography of blood plasma of Pyura stolonifera.

Incubation experiment	Fraction 9	Fraction 10	Fraction 11	Fraction 12
Natural	0.5 (275, 327)	0.8 (275, 327)	1.3 (273, 325)	1.7 (271, 325)
24 hr ^{59}Fe	0.7 (272, 325)	0.7 (272, 323)	0.9 (272, 323)	1.3 (268, 322)
54 hr ^{59}Fe	0.6 (273, 327)	0.5 (273, 327)	0.6 (273, 326)	0.8 (273, 325)
120 hr ^{59}Fe	0.5 (275, 329)	0.5 (272, 327)	0.7 (273, 327)	1.3 (273, 325)
24 hr ^{54} Mn	0.8 (278, 324)	0.6 (285, 324)	0.6 (282, 323)	0.9 (280, 322)
98 hr ^{54}Mn	0.4 (285, 327)	0.4 (275, 327) (283)	0.6 (274, 326) (282)	0.8 (275, 326) (282)
^{54}Mn in plasma	0.3 (273, 323)	0.6 (273, 323)	0.5 (273, 323)	—

g and A are the gyromagnetic ratio and hyperfine splitting constant, respectively. When this band was separated into the "327 nm" compounds and the "275 nm" insoluble fraction, a relatively weak Mn(II) signal was obtained for the former, but no signal was observed for the latter. As mentioned above, the isolated insoluble fraction turned purple on standing, and it was this sample that had been studied. The loss of most of the Mn(II) signal when the "330" band was separated into its components could be due to the Mn(II) being oxidized during the change in color of the insoluble fraction.

In ^{59}Fe and ^{54}Mn experiments with *P. stolonifera*, the metals affected the spectral characteristics of the various fractions throughout the N-acetylaminosugar band. The data for the fractions immediately on elution from the column are presented in Table III. The results show that with ^{59}Fe the largest effect on the positions of the bands was after 24-hr incubation, where both bands shifted to about 3 nm lower wavelength. The 24-hr ^{54}Mn whole-animal incubation also shifted the "327 nm" band to lower wavelength by about 3 nm, but the "275" band moved up to 10 nm higher wavelength, and with 98-hr incubation a new shoulder appeared at about 282 nm. In the ^{54}Mn plasma-incubation experiment, little variation in the ratio of the absorption bands was observed for fractions 9, 10, and 11, and no variation in the absorption maxima that were in similar positions to the bands in the 24-hr ^{59}Fe experiment. For *A. ceratodes* the absorbance maximum of fraction 9, normally at 314 nm, was transformed to 325–330 nm by incubation of the animal with ^{59}Fe, and to a wavelength lower than 314 nm by incubation with ^{54}Mn. This effect was not reproduced by direct interaction of these metals with the plasma.

When specimens of *P. stolonifera* were exposed for 24 and 72 hr to sea water containing an aquahydroxo complex of ^{51}Cr(III), the chromium was taken into the plasma, but this activity was lost upon dialysis. The failure of ^{51}Cr to bind to any of the plasma fractions could be due to the inertness of Cr(III) to substitute reactions, in contrast to the other metal ions.

The uptake of ^{48}V by whole animals and plasma alone was studied. Both species' plasma had ^{48}V activity. However, all activity was dialyzed from *P. stolonifera* plasma, showing that there is no strong binding between vanadium and the plasma components of this species. Dialysis experiments with *A. ceratodes* were not conducted. The plasma was chromatographed and had activity in fractions 1–2 and 8–13 corresponding to the two main chromatography bands detected by UV-visible spectroscopy. Sea water innoculated with ^{48}V (V) and chromatographed gave activity in fractions 8–13. Therefore, it is possible that the ^{48}V in the plasma is not bound to the aminosugar component. In a gel electrophoresis experiment, the majority of the activity traveled with the front, showing that most of the vanadium in the plasma is not bound to the components, but probably is present as a low molecular weight, negatively charged compound such as $H_2VO_4^-$, present in water at this pH.

Discussion

Plasma spectra (Fig. 1) are similar for *P. stolonifera* and *A. ceratodes*, iron-containing and vanadium-containing ascidians respectively. Both spectra have shoulders or bands in the 260–275 and 300–330 nm regions. The main difference between the two species is the presence of pink compound(s) having maxima at 275 and 497 nm in the *P. stolonifera* plasma. The spectrum of plasma of *A. nigra*, a species in the same genus as *A. ceratodes*, has shoulders in the 260–275 and 300–330 nm regions, and at about 380 nm (Kustin *et al.*, 1976). Thus there appears to be some correlation between the plasma spectra for *A. ceratodes* and *A. nigra.* We have observed that lysing of blood cells can cause a transitory absorbance in the 380 nm region in the plasma.

Elution of the plasma of *P. stolonifera* and *A. ceratodes* from Sephadex G75 results in similar patterns for absorbance vs. fraction collected (Fig. 2). Tests of the fractions for proteins and N-acytylaminosugars show 1–2 and 3–5 to be proteins, and 8–13 to be N-acytylaminosugar for both species.

Gel electrophoretic patterns of the plasma of *A. ceratodes* and *P. stolonifera* (Fig. 4) confirm the above mentioned similarities between the plasma of the two species. The proteinaceous materials are contained in earlier elution fractions 1–2 and 3–5 and show dissimilar electrophoretic patterns between species (Fig. 4). The last compounds eluted from Sephadex G75 for both species (fractions 8–13, Fig. 2) test as N-acetylaminosugars and show no protein. The fact that there is a single band in the gel electrophoretic patterns of fractions 9 is not inconsistent with the possible presence of two closely related compounds or two compounds that interconvert, as is suggested by the variations of the absorption ratio (275/325 nm) with increasing fraction (8–13). In any case the two compounds referred to above are saccharides and the compound(s) having an absorption in the 300–330 nm region test as N-acetylaminosugars.

The gel electrophoretic pattern for *A. ceratodes* (A, B, Fig. 4) suggests equilibria between some proteinaceous compounds. This may be the basis of the changes in the plasma composition noted in this species. The difference between the two patterns is the disappearance of the two bands between 0.50–0.55 and the appearance of single bands at 0.35 and 0.91, suggesting as one explanation that two proteins of intermediate molecular weight can yield proteins of higher and lower molecular weight or *vice versa.* The appearance of a 0.50–0.55 band in fraction 5B from a plasma sample containing only a 0.35 band indicates that the conversion can take place outside the animal.

The CD spectra of the pink compound contained in Sephadex fractions 3–5 from *P. stolonifera* dominates the CD spectrum of the plasma of this species. This pink compound is not present in *A. ceratodes*, but compounds with similar spectra occur in the plasma of other species in the genus *Pyura* (unpublished results, J. H. Swinehart). The role of these compounds in the plasma is not yet understood.

The N-acetylaminosugars found in fractions 8–13 of the elution patterns from Sephadex G75 (Fig. 2), and represented by the spectra in Figure 3B, appear to be important in association with metal ions. The spectral properties of these compounds also reflect the metal association. Figure 3 shows that the compounds isolated from these fractions for *P. stolonifera* and *A. ceratodes* have maxima at 327 and 314 nm, respectively. If *A. ceratodes* is incubated in ^{59}Fe (or Fe) the 314 nm maxima shifts to 325–330 nm, while incubation with ^{54}Mn (Mn) shifts the maxima below 314 nm. Incubation of *P. stolonifera* with ^{54}Mn results in a shift of the 327 nm band to shorter wavelengths, and ^{59}Fe incubations a shift to slightly longer wavelengths.

Thus, on the basis of the metal-induced spectral changes, metal analyses, and electron-spin-resonance studies carried out on these compounds it seems reasonable to focus on them as possible metal carriers in the plasma. Accordingly, animals were incubated in various metal radiotracers, and plasma eluted from Sephadex G75.

Whole-animal ^{59}Fe uptake into the plasma of *P. stolonifera* (Fig. 5) shows associations with all classes of compounds eluted from Sephadex G75. This distribution occurs at both high and low ^{59}Fe levels. Very little can be done to correlate the absolute activity with the sequence of Fe transfer in the plasma because each elution experiment represents an animal, and as the experiment proceeds the animal tends to degenerate so that uptake requirements and plasma chemistry may vary with time for a particular animal. However, comparison of whole animal data with that for uptake of ^{59}Fe by the plasma itself shows that only in whole animal incubation is ^{59}Fe incorporated in the "pink" compound in fractions 3–5. If *A. ceratodes* is incubated at low ^{59}Fe levels (0.1 μCi in 4 l sea water) incorporation occurs only into the band contained in fractions 8–13. At higher levels of ^{59}Fe, *A. ceratodes* incorporates the metal into all classes of bands eluted from Sephadex G75.

Thus it appears that for *A. ceratodes*, in contrast to *P. stolonifera*, there is a preferred uptake of Fe in fractions 8–13 and, if sufficient Fe is present, it is indiscriminately removed by several components of the plasma. This difference in the uptake of ^{59}Fe by the two species could be pertinent to the difference in their ability to concentrate iron in the blood cells.

The ^{54}Mn uptake experiments show that there is not a rapid equilibration of manganese between sea water and plasma. If such an equilibration were present, a rapid increase in the activity of the plasma followed by a close correspondence to the sea-water activity curve should occur. The maximization and subsequent decrease in activity of the plasma can result from slow re-equilibration with the sea water, uptake by other organs, and uptake by the blood cells. For both species the ^{54}Mn activity of the blood cells increases over the entire course of the experiments, and in the case of *P. stolonifera*, an iron-containing fraction from the blood cells, so named "ferriascid," has been isolated and shows increased ^{54}Mn activity, as do the cell residues.

Manganese-54 uptake by both species results in the incorporation of the isotope in fractions 8–13 of the elution pattern from Sephadex G75. This incorporation was substantiated by the observation of the Mn(II) ESR signal in samples of plasma from *P. stolonifera* eluted from Sephadex G75. For both ^{59}Fe and ^{54}Mn, dialysis of these plasma fractions does not result in a loss of the metal. It is concluded that ^{59}Fe and ^{54}Mn are bound to the N-acetylaminosugar(s) in these fractions.

The result of ^{48}V uptake for *A. ceratodes* can be interpreted in terms of ^{48}V binding to the proteins in fraction 1–2, but it is unclear whether the activity in fractions 8–13 is completely due to free vanadium species or partly to ^{48}V bound to the N-acetylaminosugar compound.

A major difference between the two species is that the proteins in fractions 1–2 bind vanadium even at low concentrations in *A. ceratodes*, a species that concentrates vanadium in its blood cells, but not in *P. stolonifera*. The reverse was found to be true for ^{59}Fe. In *P. stolonifera*, a species that concentrates iron in its blood cells, the proteins in fractions 1–2 were found to bind iron at both high and low concentrations, but in *A. ceratodes* at low concentrations no iron was detected in these fractions.

In summary, studies with ^{59}Fe, ^{54}Mn, ^{51}Cr and ^{48}V show that the chemistry of the sea water-plasma interface does not discriminate between these elements, but that selectivity occurs during the binding of the plasma components to the metals. The N-acetylaminosugar compounds bind ^{54}Mn and ^{59}Fe in both species, and possibly ^{48}V in *A. ceratodes* . The proteins in fractions 1 and 2 bind ^{59}Fe in both species at high concentrations but only in *P. stolonifera* at low concentration, and

^{48}V only in *A. ceratodes* at either high or low concentrations. The proteins in fractions 3–5 bind ^{59}Fe in *P. stolonifera.*

ACKNOWLEDGMENTS

The authors wish to thank Dr. R. Bramley of the Research School of Chemistry of the Australian National University for the ESR measurements, Dr. Patricia Mather of the Queensland Museum for useful discussions, and the University of Queensland for financial support for Professor J. H. Swinehart during his stay at the University on sabbatical leave from the University of California, Davis. Research support is acknowledged from the Australian Research Grants Committee (C. J. Hawkins) and the Research Corporation (J. H. Swinehart).

LITERATURE CITED

ALLAN, J. E., 1959. The determination of iron and manganese by atomic absorption. *Spectrochemica Acta,* **15**: 800–806.

BIELIG, H. J., K. PFLEGER, W. RUMMEL, AND E. SEIFEN, 1961. Aufnahme und Verteilung von Vanadin bei der Tunicate *Ciona intestinalis* L. *Hoppe-Seyler's Z. Physiol. Chem.,* **326**: 249–258.

BIGGS, W. R., AND J. H. SWINEHART, 1976. Vanadium in selected biological systems. Pp. 141–195 in H. Siegel, Ed., *Metals in Biological Systems.* M. Dekker, Inc., New York, New York.

CARLISLE, D. B., 1968. Vanadium and other metals in ascidians. *Proc. R. Soc. Lond. Ser. B.,* **171**: 31–42.

CHAN, S. I., B. M. FUNG, AND H. LÜTJE, 1967. Electron paramagnetic resonance of Mn(II) complexes in acetonitrile. *J. Chem. Phys.,* **47**: 2121–2130.

COLLINS, P. F., H. DIEHL, AND G. F. SMITH, 1959. 2,4,6-Tripyridyl-s-triazine as a reagent for iron. *Anal. Chem.,* **31**: 1862–1867.

ENDEAN, R., 1955a. Studies of the blood and tests of some Australian ascidians. I. The blood of *Pyura stolonifera* (Heller). *Aust. J. Mar. Freshw. Res.,* **6**: 35–39.

ENDEAN, R., 1955b. Studies of the blood and tests of some Australian ascidians. III. The formation of the test of *Pyura stolonifera* (Heller). *Aust. J. Mar. Freshw. Res.,* **6**: 157–164.

KUSTIN, K., D. S. LEVINE, G. C. MCLEOD, AND W. A. CURBY, 1976. The blood of *Ascidia nigra:* Blood cell frequency distribution, morphology, and the distribution and valence of vanadium in living blood cells. *Biol. Bull.,* **150**: 426–441.

LEVINE, E. P., 1962. Studies on the structure, reproduction, development and accumulation of metals in the colonial ascidian *Eudistoma ritteri* Van Name, 1945. *J. Morphol.,* **111**: 105–137.

MEIROVITCH, E., AND A. LANIR, 1978. Electron spin resonance of manganese(II) complexes with proteins. *Chem. Phys. Letters,* **53**: 530–535.

NODDACK, I., AND W. NODDACK, 1940. Die Haüfigkeiten der schwermetalle in Meerestieren. *Ark. Zool.,* **32**: 1–35.

PEARSE, A. G. E., 1960. *Histochemistry: Theoretical and Applied,* 2nd Ed., J. & A. Churchill Ltd., Lond. 998 pp.

REISSIG, J. L., J. L. STROMINGER, AND L. F. LELOIR, 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. *J. Biol. Chem.* **217**: 959–966.

SWINEHART, J. H., W. R. BIGGS, J. D. HALKO, AND N. C. SCHROEDER, 1974. The vanadium and selected metal contents of some ascidians. *Biol. Bull.,* **146**: 302–312.

Reference: *Biol. Bull.*, **159**: 669–680. (December, 1980)

CHEMISTRY OF THE BLOOD OF THE ASCIDIAN *PODOCLAVELLA MOLUCCENSIS*

CLIFFORD J. HAWKINS, DAVID L. PARRY, AND CRAIG PIERCE

Department of Chemistry, University of Queensland, Brisbane, Australia 4067

ABSTRACT

The blood plasma of the aplousobranch *Podoclavella moluccensis* was found to have features similar to the plasma of ascidians from different suborders, including the presence of a yellow saccharide. The plasma concentrates a number of transition metals, especially vanadium, manganese, zinc, and chromium. The dominant blood cells were yellow morula cells and blue granular cells. A yellow ethanolic extract of the cells had a visible spectrum similar to a β-carotene with an additional intense absorption at about 270 nm. Magnesium and a trace of iron(III) were the only metals detected in that fraction. A subsequent purple acidified-ethanol extract, also possibly a carotenoid, and the cell residue contained a number of metals. Relative to the plasma, these two fractions concentrated calcium, and also iron, vanadium, and chromium, to a much greater extent than magnesium. This is the first report of chromium in the blood cells of ascidians or of significant concentrations of zinc in the plasma. The vanadium was not present as V(III) in the morula cells, as it is in some phlebobranchs, but appears to be in the granular cells, and the cell residue gave an ESR signal characteristic of VO^{2+}. The UV-visible and circular dichroism spectra of the various blood fractions are reported and compared to similar fractions from the aplousobranch *Sigillina cyanea* and the stolidobranch *Polycarpa pedunculata*.

INTRODUCTION

A companion paper from this laboratory compares the chemistry of the blood plasma of two ascidians: *Pyura stolonifera* from the suborder Stolidobranchia, and *Ascidia ceratodes* from the suborder Phlebobranchia (Hawkins *et al.*, 1980). Plasma components were separated by gel chromatography and gel electrophoresis, and uptake of metals by these components was studied using radiotracers with whole animals and plasma. Besides proteins, each species has in its plasma an N-acetyl-aminosugar compound that has different spectroscopic characteristics for the two species and that takes up iron and manganese in both, and possibly vanadium in *Ascidia ceratodes*. Aminosugar compounds have also been isolated from the blood cells of these two species (Biggs, 1977; Dorsett, Hawkins, Merefield, Parry, and Stern, in preparation).

Except for metal concentrations, little has been published concerning the chemistry of blood from the third suborder, Aplousobranchia. Relatively large vanadium and iron concentrations have been found in certain species (Biggs and Swinehart, 1976). In contrast to the phlebobranchs, in which the vanadium is in the (III)

Received July 20, 1979; accepted August 20, 1980.

Abbreviations: Circular dichroism (CD), periodic acid Shiff (PAS), inductively-coupled plasma optical emission (ICP), gyromagnetic ratio (g).

oxidation state localized in morula "green cells" called vanadocytes, the aplousobranchs have vanadium in the (IV) oxidation state and its location is unknown.

In this study, concentrations of metals in various fractions of the blood were determined for the aplousobranch *Podoclavella moluccensis* (Sluiter, 1904), which belongs to the family Clavelinidae. The UV-visible absorption and circular dichroism (CD) spectra of the fractions were measured and compared with spectra of similar fractions from other ascidians. Electron spin resonance studies were carried out to determine the oxidation states of the metals.

Materials and Methods

Ascidians were collected in the Capricorn Reefs in the vicinity of Heron Island, Queensland, Australia, in late spring, 1978, and were identified by Dr. Patricia Mather (Kott) of the Queensland Museum. Blood collection and fractionation were carried out at the Heron Island Research Station. Two species of the suborder Aplousobranchia were studied: *Podoclavella moluccensis,* collected from a rubble substrate at about 15 m, and *Sigillina cyanea,* (*Colella cyanea,* Herdman, 1899; *Eudistoma cyanea,* Kott, 1957) collected from a sandy bottom at 30 m. Two distinct forms of the stolidobranch *Polycarpa pedunculata* (Heller, 1878) were collected. One, found both at 30 m on a sandy bottom and at about 8 m attached to the reef rock on the drop-off, had a bright orange test and blood. The other, collected from reef rock at 8 m, had a whitish test with yellow blood. Following collection, the ascidians were kept in aquaria in running sea water.

The blue blood of *Podoclavella moluccensis* was extracted within 24 hr of collection from individual stolons of the zooids, using a syringe. The blood was centrifuged and the cells mechanically lysed in 90% ethanol, giving a bright yellow solution. Extraction of the cells with ethanol was repeated until a clear solution was obtained. The residue was then exhaustively extracted with 0.3% HCl in ethanol, giving a purple solution. The blue cell residue and both extracts were stored at 4°C, and the pale yellow plasma was stored frozen.

Sigillina cyanea zooids have a common stolon. Blood was removed from this species and from the solitary ascidian *Polycarpa pedunculata* by cutting the stolons near the base, creating a well when the animals were inverted, and by sucking the blood from this well with a syringe.

A sample of plasma from a colony of *Podoclavella moluccensis* was dialyzed against distilled water for 1 hr and chromatographed through Sephadex G75 with distilled water as eluent. The fractions were monitored by measuring absorbance at 275 and 325 nm. Individual fractions were tested for proteins by the biuret test, and for saccharides by the PAS method (Pearse, 1968).

Metal ion concentrations in the various blood fractions were determined by inductively-coupled plasma optical emission (ICP) using a Lab Test Multielement Analyser. The ethanolic solution samples were evaporated to dryness under vacuum at room temperature and the aqueous solutions including the plasma were freeze-dried prior to digestion with HNO_3-$HClO_4$.

Spectroscopic studies were carried out with a Cary Model 17 spectrophotometer for the UV-visible absorption spectra, a Jobin Yvon Mark III Dichrograph for CD spectra, and a Varian V-4502-11X band spectrometer equipped with a multipurpose cavity operating at 9.104 GHz for electron-spin-resonance measurements.

Cells were stained both in whole blood and after fixing with 5% formalin in sea water. The stains were as follows: oil red, neutral red, methyl red, osmium tetroxide, Turnbull's iron(II), and Perl's iron(III).

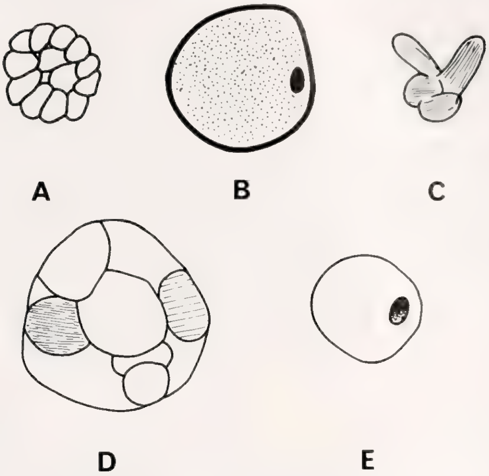


FIGURE 1. Blood cells, showing relative diameters. Approximate relative populations are given below in parentheses. (A) Mature morula cell: *Podoclavella moluccensis,* yellow (50%); *Sigillina cyanea,* yellow (60%); *Polycarpa pedunculata* (white test), yellow (80–90%); *Polycarpa pedunculata* (orange test), orange (80–90%). (B) Non-acidic granular cell: *Podoclavella moluccensis,* blue with Brownian granules (30–40%); purple (2%). (C) Purple-black globular cell: *Sigillina cyanea* with Brownian granules (30–35%). (D) Macrophage with ingested cells: each species (2%). (E) Lymphocyte: each species (10%).

RESULTS

Blood cells

Morula cells (Fig. 1) were the predominant blood cells in each of the species studied. In *Podoclavella moluccensis, Sigillina cyanea,* and the specimens of *Polycarpa pedunculata* with white test, these cells were yellow and made up 50, 60, and 80–90%, respectively, of the cell populations. The orange form of *Polycarpa pedunculata* had bright orange morula cells (80–90% of total population) which readily agglutinated, and the blood in the test of this form went black on relatively brief exposure to air. Globules within the yellow morula cells from specimens of *Podoclavella moluccensis* and *Sigillina cyanea* gave positive acid tests with oil red, neutral red, and methyl red, although the whole blood was neutral. No staining tests were conducted with blood from specimens of *Polycarpa pedunculata.* The amount of acid in the blood cells from the species *Podoclavella molluccensis* must be small because lysis in water (freezing was necessary for lysis) and in alcohol gave a neutral solution. A non-acidic blue granular cell was the second most predominant cell type for *Podoclavella moluccensis* (30–40%). Non-acidic purple granular cells (about 2%) were also present. For *Sigillina cyanea* the second major cell type (30–35%) contained large purple to black globules. Lymphocytes (< 10%) and some large macrophages were also present in each species. The cells are diagramatically represented in Figure 1.

Cells from specimens of *Podoclavella moluccensis* were stained for vanadium and iron. Osmium tetroxide, known to stain cells containing V(III) black (Henze, 1913), and cell membranes containing fats grey (Pearse, 1968), stained the morula cells grey, suggesting that the vanadium found in the blood is not in the V(III) form. Turnbull's solution turned the morula cells a bright turquoise blue, indicating the presence of Fe(II). A negative test was obtained with Perl's stain for Fe(III) although the blue granular cells took on a more purplish tint.

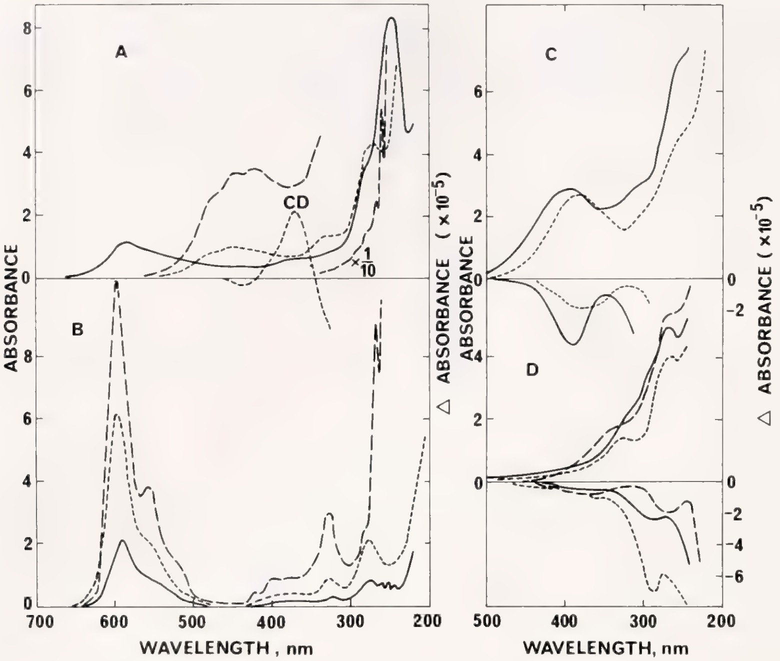


FIGURE 2. Absorption and CD spectra of blood fractions. (A) from blood cells of *Podoclavella moluccensis*: ethanol extract (dotted line); hexane solution of solid from ethanol extract (dashed line); and acidified ethanol extract (solid line). (B) from blood cells of *Sigillina cyanea*: ethanol extract (dotted line); hexane (dashed line) and 85% aqueous methanol (solid line) solutions of the blue solid isolated from the ethanol extract. (C) from blood cells of *Polycarpa pedunculata* (orange form): ethanol (dotted line) and acidified ethanol (solid line) extracts. (D) plasma of *Podoclavella moluccensis*, (dotted line), and orange (dashed line) and white (solid line) forms of *Polycarpa pedunculata*. Cell pathlength 1 cm; dilution shown where applicable.

The animals were collected late in spring, when specimens of *Podoclavella moluccensis* were very active in producing larvae. It is possible that this could have affected the relative cell populations and perhaps the blood chemistry of this species.

Fractions isolated from blood cells

Blood cells of *Podoclavella moluccensis* did not lyse in water unless the water was frozen, nor did they lyse in dilute acid. Ethanol, on the other hand, caused rapid lysis of cells, giving a bright yellow extract with the absorption and CD spectra shown in Figure 2. After evaporation to dryness, the yellow fraction was partitioned between hexane and 85% aqueous methanol. It dissolved completely in the hexane layer giving the spectrum in Figure 2, and did not change color on acidification. It gave an ESR spectrum at 77 K with resonances at gyromagnetic ratio (g) values of 2.00 and 3.78 (Fig. 3). After exhaustive extraction with ethanol, the residue was extracted with acidified ethanol, giving a deep purple extract with the absorption spectrum shown in Figure 2 but no observable CD nor ESR spectra. The blue cell residue had a strong ESR resonance of g 2.01 with a fine structure characteristic of VO^{2+} (Fig. 3).

Sigillina cyanea blood cells lysed in ethanol gave a bright blue lysate with the spectrum shown in Figure 2. After exhaustive extraction with ethanol, no material was extracted into acidified ethanol. The ethanol extract gave a blue solid on evaporation to dryness. Upon partitioning between hexane and 85% methanol, 75% of this solid went into the hexane and the remainder into the methanol. The spectra of these two solutions are given in Figure 2. The blue extract had no detectable CD.

Cells of the orange form of *Polycarpa pedunculata* were also lysed in ethanol and exhaustively extracted with that solvent, giving a bright yellow solution. Subsequent extraction with acidified ethanol also gave a yellow solution. Figure 2 shows the absorption and CD spectra of both fractions. Not enough blood was isolated from specimens of *Polycarpa pedunculata* with white tests for these types of studies.

Blood plasma

Absorption and CD spectra of the plasma of *Podoclavella moluccensis* and the two forms of *Polycarpa pedunculata* are shown in Figure 2. Unfortunately the *Sigillina cyanea* plasma was destroyed before its spectrum was measured. The elution pattern of *Podoclavella moluccensis* plasma chromatographed through Sephadex G75 is given in Figure 4. A colorless protein band that gave a positive biuret test but a negative PAS test for saccharide was eluted in the first 10 ml fraction following the exclusion volume of the column. Fractions 2 and 3, part of the same band as fraction 1, were yellow and tested positive for protein and negative for saccharide. Fractions 9–12 came under the second major elution band. They were yellow and tested negative for protein but positive for saccharide. The absorption spectra for the individual fractions under this chromatographic band varied across the band (Fig. 5). The CD spectrum for fraction 10 is included in Figure 5.

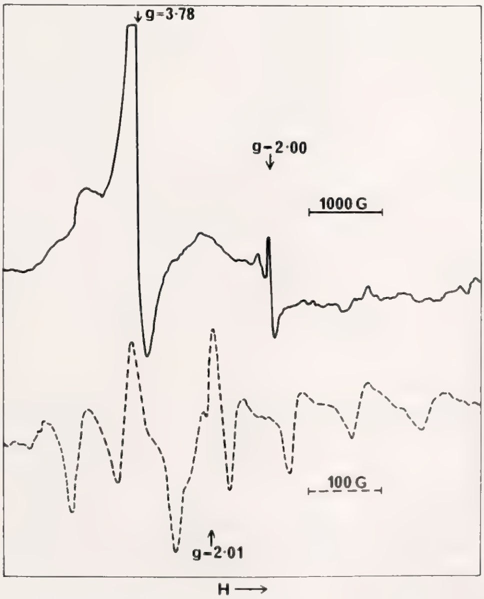


FIGURE 3. ESR spectra of the ethanol extract of the blood cells (solid line) and the cell residue (dashed line) of *Podoclavella moluccensis*.

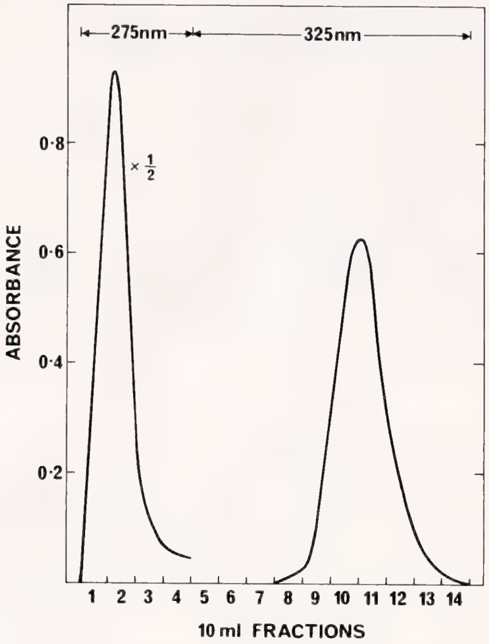


FIGURE 4. Elution pattern of plasma of *Podoclavella moluccensis* through Sephadex G75 with water as eluent.

Metal concentrations in blood fractions

The various fractions of the blood of *Podoclavella moluccensis* were analyzed by ICP for Ca, Mg, V, Cr, Mn, Fe, Cu, and Zn. In Table I, the results are compared

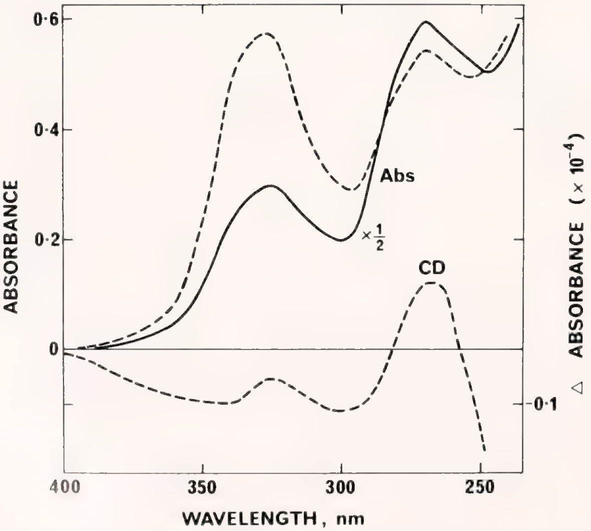


FIGURE 5. Absorption spectra of fractions 10 (dashed line) and 11 (solid line) and the CD spectrum of fraction 10, from the chromatographic separation of the plasma of *Podoclavella moluccensis*. Cell pathlength 1 cm; dilution shown where applicable.

with the mean of the limits of the range of concentrations found in sea water (Parker, 1972).

Discussion

The visible absorption spectrum of the yellow fraction extracted from *Podoclavella moluccensis* blood cells possessed a broad band centered at 450 nm with shoulders at 470 and 428 nm, very similar to the corresponding band in the β-carotene spectrum (Vetter *et al.,* 1971). However, the yellow fraction had more intense absorption in the UV than β-carotene, and had a band at 270 nm, as is found in spectra of carotenoproteins such as ovorubin (Cheesman, 1958), However, carotenoproteins are usually not soluble in ethanol, as the protein moiety is denatured by the solvent. The result of partitioning between hexane and 85% methanol was characteristic of an epiphasic carotene, and not of a carotenoprotein. Carotenoids have been isolated previously from a number of ascidians in the three suborders (Goodwin, 1952).

The acidified-ethanol extract's spectrum had an absorption maximum at 584 nm with shoulders on the low wavelength side of the band. Further shoulders are observed at about 360 and 280 nm with a large peak at 250 nm. On evaporation, this compound changed to a reddish-orange solid, which dissolved in acidified ethanol to give a spectrum with a maximum at 475 nm, shoulders on the high wavelength side of the band and at 355 and 280 nm, and a peak at 248 nm. The purple compound is possibly also a carotenoid, as dodecapreno-β-carotenes, for example, are known to absorb at 589 ± 14 nm (Vetter *et al.,* 1971). The spectrum also had a shape similar to those of a number of carotenoproteins (Lee, 1977) but its solubility in acidified ethanol was not consistent with its being a protein.

The UV-visible spectrum of the blue ethanol extract of *Sigillina cyanea* blood cells had an intense peak at 593 nm with a shoulder at 555 nm, a broad band at 375 nm, and peaks at 327 and 277 nm (Fig. 2). Absorptions in the visible spectrum were similar to those of the purple acidified-ethanol extract from specimens of *Podoclavella moluccensis.* Both extracts had no detectable CD spectrum. The absorption spectrum of the extract from specimens of *Sigillina cyanea* again suggested a carotenoid, and the partitioning between hexane and 85% methanol was consistent with a xanthophyll (Liaaen-Jensen, 1971).

The absorption and CD spectra of the ethanol and acidified-ethanol extracts of the cells from the orange form of the stolidobranch *Polycarpa pedunculata* (Fig. 2) were similar to one another, but completely different from the spectra of the fractions from the two aplousobranchs. The ethanol extract had a peak at 380 nm and a shoulder at 250 nm in its absorption spectrum, and a negative Cotton effect at 380 nm with a negative shoulder at 280 nm in the CD spectrum. The acidified-ethanol

Table I

Metal concentrations in blood fractions of Podoclavella moluccensis *expressed in μg/g dry weight of fraction. Sea water concentrations (μg/l) are the mean of the concentration-range limits (Parker, 1972).*

Fraction	Ca	Mg	V	Cr	Mn	Fe	Cu	Zn
Plasma—whole	1,115	1,720	4.17	0.35	0.45	1.31	0.66	4.37
Plasma—Fr. 9-12	19,980	46,310	96	940	45	4,530	130	370
EtOH extract	0	6,050	0	0	0	0	0	0
EtOH/HCl extract	2×10^5	460	480	58	3	624	18	66
Cell residue	7,000	950	681	22	7	964	16	49
Sea water	0.4×10^6	1.3×10^6	2.4	1.3	0.7	30	13	14

extract's spectrum had a peak at 396 nm and shoulders at 340, 305, and 250 nm. Its CD spectrum had a negative Cotton effect at 390 nm. Visible absorption bands of both fractions lacked the fine structure characteristic of the carotenoids' main visible absorption band as found for extracts from the aplousobranchs. The visible CD band was negative for both extracts from *Polycarpa pedunculata,* whereas the ethanol extract from *Podoclavella moluccensis* had a small negative Cotton effect at 439 nm and a large positive at 371 nm, and the blue-purple fractions from the two aplousobranchs had no detectable CD.

As has been found previously for the plasma of *Ascidia nigra* (Kustin *et al.,* 1976), *Ascidia ceratodes,* and *Pyura stolonifera* (Hawkins *et al.,* 1980), the absorption spectra of the plasma of *Podoclavella moluccensis* and the two forms of *Polycarpa pedunculata* (Fig. 2) had bands in the 300–330 and 260–275 nm regions. The spectrum for the form of *Polycarpa pedunculata* with white tests tailed further into the visible than the spectrum for the orange form. This absorption gave the plasma a pink color like that observed for another stolidobranch, *Pyura stolonifera,* with a definite absorption band at 500 nm in the spectrum of its plasma (Hawkins *et al.,* 1980). The CD spectra of the plasma of *Podoclavella moluccensis* and the two forms of *Polycarpa pedunculata* had negative Cotton effects with maxima between 360 and 380 nm (Fig. 2), as has also been found for *Pyura stolonifera.* However, for the first three of these species, the plasma had a negative Cotton effect with a maximum in the region 270–290 nm, whereas the plasma for *Pyura stolonifera* has a positive in this region.

The elution pattern for the plasma of *Podoclavella moluccensis,* chromatographed through Sephadex G75 (Fig. 2), was very similar to that for the *Pyura stolonifera* plasma with water as eluent (Hawkins *et al.,* 1980). A major protein band was eluted following the exclusion volume of the column. A colored compound was eluted on the side of this band. This was yellow for both *Podoclavella moluccensis* and *Ascidia ceratodes,* with an absorption peak at 275 nm and a tail in the visible. The corresponding fractions for *Pyura stolonifera* were pink, with absorption bands at 497 and 275 nm. The elution of a saccharide in the second major chromatographic band was also consistent with the findings for *Pyura stolonifera* and *Ascidia ceratodes.*

For these latter two species the saccharide was identified as an N-acetylaminosugar, but insufficient material for this test was available from *Podoclavella moluccensis.* The absorption spectra for the individual fractions for each of the three species varied across the chromatographic band, showing that at least two compounds were present (Fig. 5). The ratio of the absorption peaks' intensities (270:327 nm) increased across the chromatographic band. For *Pyura stolonifera* and *Ascidia ceratodes* the two compounds were separated. The yellow compound had an absorption maximum at 314 nm for *Ascidia ceratodes* and at 327 nm for *Pyura stolonifera.* From the spectra in Figure 5 it would appear that the *Podoclavella moluccensis* yellow compound also absorbed at 327 nm. Its CD spectrum was also very similar to the corresponding compound from *Pyura stolonifera,* with negative Cotton effects at 350 and 300 nm, and a positive at 268 nm (Fig. 5). The spectrum went negative below 250 nm.

Many published papers have been concerned with metal ions in ascidians, but most have dealt with the concentrations of metals in whole animals (see, for example, Biggs and Swinehart, 1976). It is difficult to evaluate from these studies whether ascidians have used the metals, or whether they are present through contamination via the ascidians' filter feeding. If the metals accumulate in the blood plasma or, more importantly, in the blood cells, there is a much greater possibility of the metals fulfilling a biological function. For this reason, the various fractions of the blood of *Podoclavella moluccensis* were analysed by ICP for a range of metals.

The concentrations of the metals in the plasma were in the order: Mg > Ca > Zn $\simeq$ V > Fe > Cu > Mn > Cr. Using Mg as the reference, the transition metal ion concentrations can be seen to have increased markedly in the plasma relative to the normal levels of dissolved metals in sea water, especially vanadium, and to a lesser extent, manganese, zinc, and chromium. This is true whether the mean value or the concentration range in sea water is used as the basis for comparison. Further, the relative concentrations in the plasma did not mirror those normally found in oceanic sea water. The apparent very marked accumulation of the above four metals in the plasma, relative to the others studied, could be a direct result of higher than normal concentrations of these metals, both dissolved and in particulate form, in the water pumped by these animals in their coral reef habitats. The present study is the first report of high zinc levels in the blood plasma of ascidians. The skeletal carbonate of the corals in the reefs of the Capricorn Group, where the colonies of *Podoclavella moluccensis* were collected, has been found to have relatively high levels of zinc (St. John, 1974). For example, in samples of *Acroporidae* 1400 ng/g Zn was found compared to 740 ng/g Fe and 210 ng/g Cu. St. John claimed that the concentrations of trace elements in the corals reflected concentrations in the water of the environment, and hence these results would indicate larger concentrations of zinc in the vicinity of the reefs compared to oceanic sea water. The high levels of zinc in specimens of *Podoclavella moluccensis* could also be a result of the ingestion of particles of coral containing high levels of zinc rather than the presence of larger than normal concentrations of dissolved zinc in the sea water. The values taken for the concentrations of manganese and vanadium in sea water might also be misleading because they refer to dissolved metals. Concentrations of manganese in particulate matter (Parker, 1972), and of vanadium in marine sediments and plankton (Biggs and Swinehart, 1976), which are known to be high, were not included. The apparent accumulation of Cr in the plasma might have a similar explanation, but in the case of Cr and also V relatively high levels of these metals were found in blood-cell fractions, and hence the levels in the plasma might not be simply a consequence of the concentrations in the animals' food intake.

The high Ca/Mg concentration ratio in the plasma (0.65) compared to sea water (0.31) might also be a consequence of the relative abundance of these elements in the food supply of species collected from coral reefs, which, of course, are composed largely of calcium carbonate. In contrast, specimens of *Phallusia mammillata* (Henze, 1912; Robertson, 1954) and *Pyura stolonifera* (Endean, 1955), collected from a rock substrate, have been found to have Ca/Mg ratios in the plasma almost identical to that in sea water. The variations found in the Ca/Mg ratios for the various blood fractions are also interesting. In the saccharide fraction from the plasma, the Ca/Mg value of 0.42 is lower than that in the whole plasma (0.65). This is somewhat surprising, as some sugars have been found to form much more stable complexes with calcium than with magnesium (Angyal, 1973). In the blood-cell fractions, no calcium was detected in the yellow ethanol extract, whereas 6050 μg/g Mg was found. In direct contrast, in the purple acidified-ethanol extract about 2×10^5 μg/g Ca and 460 μg/g Mg were found—a Ca/Mg ratio of 422. The cell residue had 7000 μg/g Ca and 950 μg/g Mg, with Ca/Mg equal to 7.4. Since the chloride and sulfate salts of calcium were soluble enough in ethanol to be extracted into ethanol, the fact that no detectable levels of Ca were found in the ethanol extract indicates that Ca was either precipitated out as an insoluble salt other than the chloride or sulfate, or was coordinated in a complex insoluble in ethanol. At least one calcium compound was soluble, however, in acidified ethanol. Perhaps Ca was bound to the purple pigment. The magnesium in the yellow ethanol extract could be free Mg^{2+} or bound to the yellow pigment.

The saccharide fraction from the plasma had high concentrations of Fe and Cr, and to a lesser extent Zn. However, a comparison of these concentrations to those in the plasma shows that only Fe and Cr were concentrated by the saccharide compound to any marked extent.

No detectable levels of the transition metals were found for the yellow ethanol extract of the blood cells by ICP, but relatively high concentrations of iron and vanadium were found in the purple acidified-ethanol extract (624 and 480 μg/g, respectively) and also in the cell residue (964 and 681 μg/g, respectively). Fe/V concentration ratios in the two fractions were similar. However, a trace of iron was present in the yellow ethanol extract, as an ESR signal typical of non-heme iron (Peisach and Blumberg, 1969) was detected. The spectrum was identical to that obtained for an iron-containing N-acetylaminosugar compound isolated from the morula blood cells of *Pyura stolonifera* (unpublished results, C. J. Hawkins and D. L. Parry). No iron or vanadium ESR signal was observed at 77 K for the purple extract shown to contain both elements. This could result from the rapid relaxation of the electron spins for the two elements. An ESR signal characteristic of VO^{2+}, detected for the blue cell residue, is similar to the spectrum obtained for V(IV) in the zooids of the aplousobranch *Eudistoma diaphanes* (Swinehart *et al.,* 1974). Superimposed on this spectrum is a relatively weak signal at about g 2.06 that could result from the iron. No attempt was made to exclude oxygen during the isolation of these fractions, and therefore it is possible that the vanadium had a lower oxidation state in whole cells than in the isolated fractions. However, a negative stain for V(III) was obtained for the whole cells.

In the purple fraction, besides Fe and V, significant concentrations of Zn and Cr were found with traces of Cu and Mn. Comparing the metal concentrations in this fraction to those in the plasma obtained the following ratios: V, 115; Cr, 166; Mn, 6; Fe, 476; Cu, 27; and Zn, 15. The same comparison for the cell residue, yields the following ratios: V, 163; Cr, 63; Mn, 16; Fe, 736; Cu, 24; and Zn, 11. From these results it can be concluded that Fe, V, and Cr accumulate in the blood cells. Chromium has been found in whole zooids (Levine, 1962) and in whole colonies (Swinehart *et al.,* 1974) of the aplousobranch *Eudistoma ritteri* and in the body tissues of *Ciona intestinalis* (Bielig *et al.,* 1961), but the present paper is the first report of Cr in ascidian blood cells. However, in terms of the actual concentrations of transition metals in the blood cells, Fe and V are the predominant metals, with an Fe/V ratio of about 1.3. Iron and vanadium are also the predominant transition metals in the blood cells of *Ciona intestinalis,* with an Fe/V ratio of 0.53 (Bielig *et al.,* 1961). The vanadium in that species, traditionally classified as a phlebobranch, also occurs in the (IV) oxidation state (Swinehart *et al.,* 1974). In the blood cells of species of the family Ascidiidae belonging to the suborder Phlebobranchia, V(III) is the predominant transition metal ion (Biggs and Swinehart, 1976), although significant concentrations of Fe have been reported for the blood cells of *Phallusia mammillata* (Bielig *et al.,* 1961) and in the blood of *Ascidia ceratodes* (Swinehart *et al.,* 1974). In the blood cells of the stolidobranch

Pyura stolonifera, no V has been detected, but relatively large concentrations of Fe have been found (Endean, 1955).

In the Ascidiidae the V(III) is localized in the morula "green" cells called vanadocytes (Biggs and Swinehart, 1976). In *Ciona intestinalis,* the vanadium is present in cells "that look somewhat different" from the vanadocytes (Rummel *et al.,* 1966). In *Podoclavella moluccensis,* vanadium is found in the purple fraction and in the blue cell residue, and not in the yellow fraction. Therefore, it is tempting to conclude that the vanadium is in the purple and blue granular cells and not in the yellow morula cells.

ACKNOWLEDGMENTS

The authors wish to thank especially Dr. Patricia Mather of the Queensland Museum for identifying the ascidians collected in the Capricorn Group and for useful discussions. The authors are also grateful to Mr. R. W. Garrett of this department for the ESR measurements, to Dr. A. J. Bruce, director of the Heron Island Research Station, for use of the facilities at the Station, and to staff and student members of the College of Idaho for assistance in the collection of the ascidians at Heron Island during their study program in Australia. Research support is acknowledged from the Australian Research Grants Committee.

LITERATURE CITED

ANGYAL, S. J., 1973. Complex formation between sugars and metal ions. *Pure Appl. Chem.,* **35**: 131–146.

BIELIG, H. J., E. JOST, K. PFLEGER, W. RUMMEL, AND E. SEIFEN, 1961. Aufnahme und Verteilung von Vanadin bei der Tunicate *Phallusia mamillata* Cuvier. *Hoppe-Seyler's Z. Physiol. Chem.,* **325**: 122–131.

BIELIG, H. J., K. PFLEGER, W. RUMMEL, AND E. SEIFEN, 1961. Aufnahme und Verteilung von Vanadin, bei der Tunicate *Ciona intestinalis* L. *Hoppe-Seyler's Z. Physiol. Chem.,* **326**: 249–258.

BIGGS, W. R., 1977. Transition metals in ascidians: The blood of *Ascidia ceratodes. Ph.D. thesis,* University of California, Davis, Calif., 151 pp. Diss. Abstr. **38**: 4799(B).

BIGGS, W. R., AND J. H. SWINEHART, 1976. Vanadium in selected biological systems. Pp. 141–195 in H. Seigel, Ed., *Metals in Biological Systems.* M. Dekker, Inc., New York.

CHEESMAN, D. F., 1958. Ovorubin, a chromoprotein from the eggs of the gastropod mollusc *Pomacea canaliculata. Proc. R. Soc. Lond. Ser. B,* **149**: 571–587.

ENDEAN, R., 1955. Studies of the blood and tests of some Australian ascidians. I. The blood *Pyura stolonifera* (Heller). *Aust. J. Mar. Freshw. Res.,* **6**: 35–39.

GOODWIN, T. W., 1952. *The Comparative Biochemistry of the Carotenoids.* Chapman & Hall Ltd., London, 365 pp.

HAWKINS, C. J., P. M. MEREFIELD, D. L. PARRY, W. R. BIGGS, AND J. H. SWINEHART, 1980. Comparative study of the blood plasma of the ascidians, *Pyura stolonifera* and *Ascidia ceratodes. Biol. Bull.,* **159**: 656–668.

HELLER, C., 1878. Beiträge zur nähern Kenntniss der Tunicaten. *Sitzber Akad. Wiss. Wein,* **77**: 83–110.

HENZE, M., 1912. Blood of ascidians. *Hoppe-Seyler's Z. Physiol. Chem.,* **79**: 215–228.

HENZE, M., 1913. Blood of ascidia. *Hoppe-Seyler's Z. Physiol. Chem.,* **86**: 340–344.

HERDMAN, W. A., 1899. *Descriptive Catalogue of the Tunicata in the Australian Museum.* T. Dobb, Liverpool, 139 pp.

KOTT, P., 1957. The ascidians of Australia. II. Aplousobranchiata Lahille: Clavelinidae Forbes and Hanly and Polyclinidae Verrill. *Aust. J. Mar. Freshw. Res.,* **8**: 64–110.

KUSTIN, K., D. S. LEVINE, G. C. MCLEOD, AND W. A. CURBY, 1976. The blood of *Ascidia nigra*: Blood cell frequency distribution, morphology, and the distribution and valence of vanadium in living blood cells. *Biol. Bull.,* **150**: 426–441.

LEE, W. L., Ed., 1977. *Carotenoproteins in Animal Coloration.* Dowden, Hutchinson & Ross Inc., New York, 395 pp.

LEVINE, E. P., 1962. Studies on the structure, reproduction, development and accumulation of metals in the colonial ascidian *Eudistoma ritteri* Van Name, 1945. *J. Morphol.*, **111**: 105–137.

LIAAEN-JENSEN, S., 1971. III. Isolation, Reactions in Carotenoids, Pp. 63–177 in O. Isler, Ed., *Carotenoids,* Birkhäuser Verlag, Basel.

PARKER, C. R., 1972. *Water Analysis by Atomic Absorption.* Varian Techtron, Australia, 78 pp.

PEARSE, A. G. E., 1968. *Histochemistry: Theoretical and Applied.* 3rd Ed. J. & A. Churchill Ltd., London, 759 pp.

PEISACH, J., AND W. E. BLUMBERG, 1969. Metalloproteins as studied by electron paramagnetic resonance. Pp. 71–96 in Teh Fu Yen. Ed., *Electron Spin Resonance of Metal Complexes,* Plenum Press, New York.

ROBERTSON, J. D., 1954. The chemical composition of the blood of some aquatic chordates, including members of the Tunicata, Cyclostomata and Osteichthyes. *J. Exp. Biol.*, **31**: 424–442.

RUMMEL, W., H. J. BIELIG, W. FORTH, K. PFLEGER, W. RÜDIGER, AND E. SEIFEN, 1966. Absorption and accumulation of vanadium by tunicates. *Protides Biol. Fluids Proc. Colloq.*, **14**: 205–210.

SLUITER, C. P., 1904. Die Tunicaten der Sibogo-Expedition. I. Die Socialen und holosomen Ascidien. *Sibogo-Exped.,* **56a**: 1–126.

ST. JOHN, B. E., 1974. Heavy metals in the skeletal carbonate of Scleractinian corals. Pp. 461–469 in A. M. Cameron *et al.*, Eds., *Proceedings of the 2nd International Coral Reef Symposium 2.* GBRC, Brisbane.

SWINEHART, J. H., W. R. BIGGS, D. J. HALKO, AND N. C. SCHROEDER, 1974. The vanadium and selected metal contents of some ascidians. *Biol. Bull.*, **146**: 302–312.

VETTER, W., G. ENGLERT, N. RIGASSI, AND U. SCHWIETER, 1971. IV. Spectroscopic Methods. Pp. 189–265 in O. Isler, Ed., *Carotenoids,* Birkhäuser Verlag, Basel.

Reference: *Biol. Bull.*, **159**: 681–691. (December, 1980)

EFFECTS OF MAGNETIC FIELDS ON REGENERATION IN FIDDLER CRABS

PAUL H. LEE[1] AND JUDITH S. WEIS[2]
Department of Zoology and Physiology, Rutgers, the State University, Newark, N. J. 07102

ABSTRACT

After autotomy, fiddler crabs (*Uca pugilator* and *U. pugnax*) were placed in magnetic fields (in the 100 Oersted range) produced by ceramic plate magnets in such a way that some crabs were in proximity to the N pole, and others to the S pole. Control crabs were several feet away from the magnets. Regardless of the number of limbs autotomized, direction of the field, species, or salinity of the water, crabs in proximity to the S pole regenerated and molted sooner than controls, and those by the N pole were delayed in relation to controls. Factors such as magnetic effects on enzymatic reactions and ionic flux, orientation of animals within the magnetic field, and production of electric currents in the limb stumps may be involved in these effects. The opposite responses to N and S poles may be due to the opposite direction of the gradient vector. Regeneration of juvenile crabs, and of adult crabs during the summer months, was not significantly affected by the magnetic fields.

INTRODUCTION

Many studies have been done on the effects of magnetic fields on biological systems. Barnothy *et al.* (1956) found a decrease in leukocyte formation in mice subjected to a strong magnetic field. This was interpreted as retardation of the mitotic rate. Mulay and Mulay (1961) found that sarcoma 37 ascites tumor cells degenerated in response to a magnetic field of 4000 Oersteds, and Malinin *et al.* (1976) found inhibition of growth in various mammalian cell lines. Barnothy (1963) found that the growth rate of mice was retarded in a static magnetic field, though Eiselein *et al.* (1961) found no such effect. Reno and Nutini (1963) and Cook *et al.* (1969) found effects on metabolic rate: Rapidly growing tissues (tumors and embryonic kidney) exhibited a decrease in oxygen consumption.

Some experiments have revealed different effects of north and south poles. Boe and Salunkhe (1963) found that tomato ripening was accelerated in a magnetic field, and that tomatoes placed by the S pole of a magnet ripened faster than those placed by the N pole. Taneeva (1978) found differences in effects of N and S poles on *Artemia* growth and oxygen uptake.

Limb regeneration in crabs involves a series of processes, starting with autotomy, and followed by growth and differentiation of a limb bud. The regenerating limb bud grows in a folded position within a layer of cuticle and unfolds when the animal molts. Peripheral nerves are necessary for normal regeneration. Since regeneration ends with ecdysis, factors which influence the molt cycle and its con-

Received October 8, 1979; accepted September 25, 1980.

Abbreviations used: R, (limb bud length ÷ carapace width) × 100; N, north; S, south.

[1] Present address: College of Medicine and Dentistry, Newark, N. J.

[2] Author to whom correspondence should be addressed.

trolling hormones can alter the rate of regeneration. Removal of eyestalks, a source of molt-inhibiting hormone, is a standard way of producing precocious molting and accelerated regeneration (Bliss, 1960). Skinner and Graham (1972) found that multiple autotomy, producing many regenerating limb buds, can also accelerate regeneration and ecdysis. Growth of regenerating limb buds in Crustacea is generally expressed in terms of "R value" which is (limb bud length ÷ carapace width) × 100 (Bliss, 1956). This is useful for comparing crabs of different sizes. Environmental factors including temperature, salinity, light, and water quality have been shown to affect regeneration and ecdysis (Bliss and Boyer, 1964; Rao, 1965; Weis, 1976a, b, 1977).

The experiments to be reported here were designed to study the effects of magnetic fields on regeneration and ecdysis in the fiddler crabs *Uca pugilator* and *Uca pugnax*.

MATERIALS AND METHODS

Crabs were collected from N. J. or Long Island, N. Y., or purchased from Gulf Specimen Co., Panacea, Fla. After autotomy (by pinching the merus) crabs were maintained in shallow artificial sea water (Instant Ocean) at 30‰ salinity and 25°C in individual plastic cups 5.0 cm in diameter at the bottom. These cups were placed by either the north (N) or south (S) pole of the magnets. The close proximity of N and S crabs assured that other environmental factors would be the same for all. Cups of control crabs were beyond the magnetic field. A piece of non-magnetized metal was placed with some controls. All animals were fed Purina Fly Chow twice a week before measurement and changing of the water. Limb buds of the first walking leg were measured under a stereomicroscope with a calibrated ocular micrometer, and values converted to R values. R values for this limb bud approach 20 prior to ecdysis.

We used ceramic chromate plate magnets 16 × 5 × 1.2 cm with the north pole on one flat surface and the south pole on the other. The measured strength of these magnets was non-uniform, but approximately 400–500 Gauss at the magnet, with a gradient of about 100 Gauss/cm close to the magnet, as measured by a Hall-effect gaussmeter. The gradient lessened further away from the magnet. The cups containing the crabs were placed next to the plate magnets, which stood on edge inside a plastic container (Fig. 1a) that was covered with a transparent cover. The inhomogenious field was about 250–50 Oersteds across the cups. Attempts to restrain crabs so they would have a permanent orientation with respect to the magnets were unsuccessful, as such animals died.

Experiment 1 was performed in the late fall 1978 on specimens of *U. pugnax* from N. J. (14 mm mean carapace width) with four limbs autotomized. Six crabs were exposed to N, six to S, and six served as controls. Experiment 2, performed in December, used specimens of *U. pugilator* from Long Island (18 mm carapace width) with two limbs autotomized. Six were exposed to N, 6 to S, and 12 served as controls. Experiment 3, performed in February, used specimens of *U. pugilator* from Florida (15 mm mean carapace width) with six limbs removed. Twelve were exposed to N, 12 to S, and 12 served as controls, all in water of 8‰ salinity. Experiment 4, performed in March, used specimens of *U. pugilator* from Florida (15 mm mean carapace width) with five limbs removed. Twelve were exposed to N, 12 to S, and 12 were controls. Experiments 5 and 6,

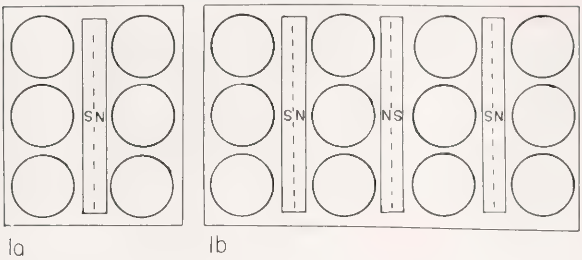


FIGURE 1. a: Arrangement of six cups by magnet for single field exposures, as in experiments 1–4. b: Arrangement of twelve cups by magnets for double field exposures, as in experiments 7–9.

done in May and June, placed specimens of Florida *U. pugilator* (18 mm mean carapace width) with seven limbs removed, in a vertical magnetic field, above and below the magnets, which were oriented horizontally. One magnet was oriented S pole upward, and another N pole up. Each was placed on top of a 8.5 cm diameter, 3.5 cm high fingerbowl containing three or four crabs. Another fingerbowl with three or four crabs was placed on top of each magnet. These crabs were therefore exposed to N or S from above or below.

In experiments 7–9 crabs were placed between pairs of magnets (Fig. 1b) so that six were exposed to N on both sides (N-N), six were exposed to S on both sides (S-S), and six were exposed to N on one side and S on the other (N-S). (This last group is comparable to most of the previous experments cited in the introduction, in which organisms were placed between the poles of horseshoe magnets.) Additional crabs were placed on the outsides of the pairs of magnets to be exposed to a single N or S field (12 crabs each), and 12 crabs served as controls. Experiment 7 was done on specimens of Florida *U. pugilator* (17 mm mean carapace width) in June, and experiments 8 and 9 on specimens of Long Island *U. pugilator* during July and August respectively. Crabs in all these experiments had six limbs autotomized.

Experiments 10–12 were done on juvenile specimens of *U. pugilator* (<13 mm carapace width). Experiment 10, done in April, exposed them to single horizontal fields, 12 crabs to N, 12 to S, and 12 controls. Experiment 11 (July) and 12 (August) exposed them to vertical fields as described in experiments 5 and 6. Sixteen crabs were exposed to N and 16 to S. Crabs in these experiments all had six limbs autotomized. Experiment 13 was done the following August (1980) on L.I. crabs (18 mm) with three limbs autotomized.

Orientation: Orientation was investigated by recording the position of regenerating crabs on either side of the magnets, or on either side of a similar sized piece of cardboard acting as a "dummy magnet." The position of each animal (three crabs in front of a N pole, three behind a N pole, three in front of a S pole, three behind a S pole, six in front of the "dummy magnet," and six behind it) was recorded every 15 min for 6-hr periods on four consecutive days. Crabs were scored as facing toward, away from, or parallel to the magnet. "Toward" and "away" were defined as within the <179° roughly facing towards or away from the magnet. Only when animals were exactly parallel to the magnet were they recorded as parallel. Care was taken not to disturb the crabs during the observation periods.

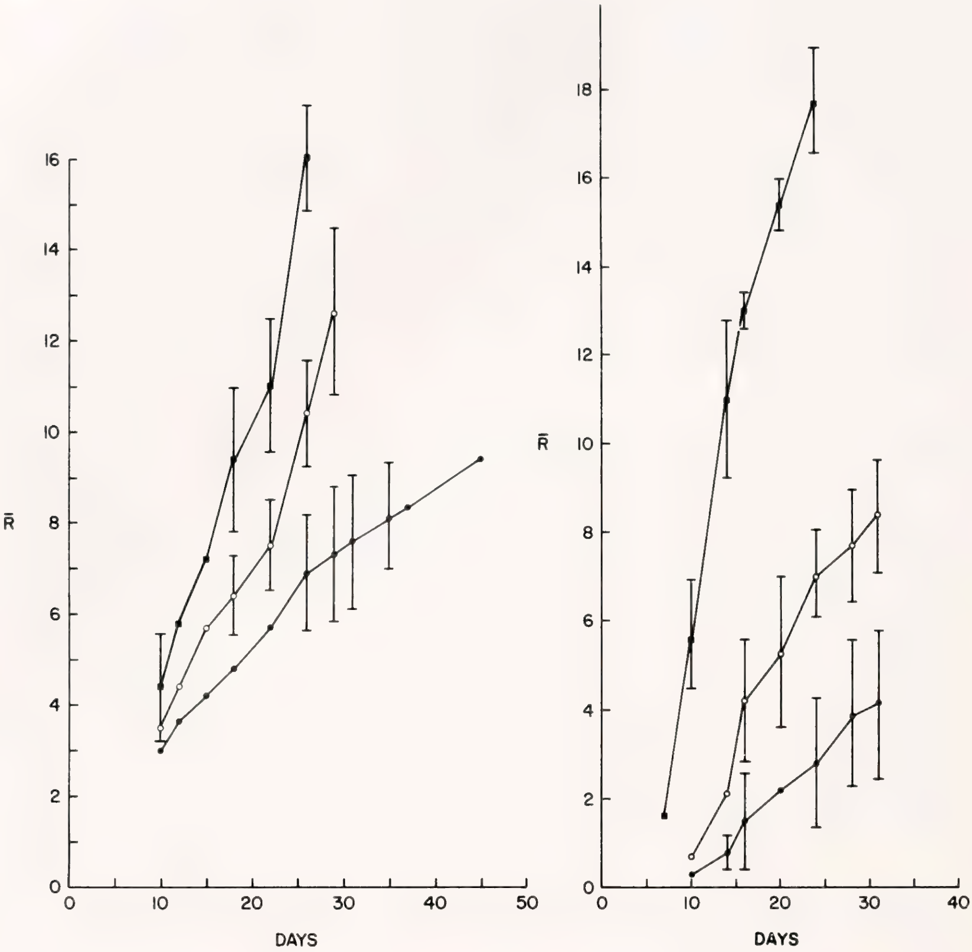


FIGURE 2. Left: Mean R values of limb buds of crabs in Experiment 1. Right: Mean R values of limb buds of crabs in Experiment 4. Open circles = controls, closed circles = adjacent to N pole, squares = adjacent to S pole. Vertical lines = standard deviations.

RESULTS

The results of experiment 1 are shown in Figure 2, left. The crabs exposed to S pole regenerated limbs at an accelerated rate compared to controls, and those exposed to N pole regenerated more slowly than controls. All animals by the S pole molted by day 29, whereas 66% of the controls molted by day 31, and 0% of crabs by N pole molted by day 31.

In experiment 2, crabs with only two limbs autotomized regenerated them much more slowly. Among the six crabs exposed to S pole, the R value at day 39 was 8.0 ± 0.8 (s.e.) and that for controls was 2.4 ± 0.5. Among those crabs by the N pole, five had not begun to regenerate, and one had reached an R value of 0.6. In experiment 3, in water of 8‰ salinity, the S pole again stimulated, and N pole retarded, growth compared to controls. For example, on day 11, the crabs at the S pole had R values of 5.2 ± 0.4 (s.e.), controls had R values of 2.4 ± 0.3, and those by the N pole had R values of 0.8 ± 0.2. In experiment 4, similar results were seen (Figure 2, right). By day 35, all of the crabs by S pole, 30% of controls, and one of the crabs by the N pole had molted. This one crab molted before its limbs were fully grown.

The vertical fields in experiments 5 and 6 produced similar effects. On day 10 in experiment 5, crabs by the S pole had R values of 8.4 ± 0.9 (s.e.) whereas those by N pole had 5.2 ± 1.1. In experiment 6, at 12 days after autotomy, the S-exposed crabs had R values of 8.3 ± 1.0, N-exposed had 4.1 ± 0.9, and controls had 6.0 ± 0.9 (s.e.).

In experiment 7, crabs exposed to N or to N-N were retarded, those exposed to S or to S-S were accelerated, and those exposed to N-S were retarded in comparison to controls. However, differences were not as great as in previous experiments, and were often not statistically significant, whereas differences in previous experiments were highly significant. For example, on day 14, R values of controls were 9.6 ± 1.4 (s.e.), those for crabs at N were 6.3 ± 1.7 (n.s.), those for N-N were 4.7 ± 1.5 (significant to 0.05), those at S were 13.6 ± 1.4 (significant to 0.05), those at S-S were 11.1 ± 1.6 (n.s.) and those at N-S were 6.8 ± 1.3 (n.s.). By day 24, 66% of S, 50% of S-S, 30% of controls, 12% of N-S, and 0% of N and N-N had molted.

When this was repeated in July (experiment 8), effects were generally reduced to non-significance, except for the retardation by N. For example, on day 10 controls had R values of 9.9 ± 0.7 (s.e.), S crabs had 9.7 ± 0.6, S-S had 10.1 ± 0.4, N crabs had 6.4 ± 0.9 (significant to 0.05), N-N crabs had 7.4 ± 1.3, and N-S crabs had 11.3 ± 1.5.

In experiment 9 (August) the same phenomenon occurred. On day 13, controls had R values of 16.8± 1.0 (s.e.), S crabs had 16.6 ± 1.2, S-S crabs had 17.0 ± 1.0, N crabs had 13.2 ± 1.3, N-N crabs had 12.5 ± 1.4, and N-S crabs had 15.6 ± 1.5. Only growth of N-N crabs was significantly different from controls. By day 17, 50% of the controls, 50% of S-S crabs, 36% of S crabs, 20% of N-S crabs, 8% of N crabs, and 0% of N-N crabs had molted. Similarly, in experiment 13, no significant differences occurred.

The experiments on the juveniles (experiments 10–12) revealed that they were unaffected by the horizontal or vertical magnetic fields. For example, in experiment 10, on day 9, R values of N-exposed crabs were 4.1 ± 0.6 (s.e.) and those of S-exposed were 4.5 ± 0.7. In experiment 11 (vertical field) on day 7, those exposed to S had R values of 7.0 ± 1.0, and those exposed to N had 5.6 ± 0.9 (s.e.). On day 6 in experiment 12 (a repeat of 11), R values for those exposed to S was 4.9 ± 0.5, and for those exposed to N were 5.1 ± 0.8. None of these are significantly different from each other. Results of all experiments are summarized in Table I.

Orientation: In the orientation study, crabs in front of the N pole were recorded as facing toward the magnet 72 times and away from the magnet 18 times (80% toward). Crabs in front of the S pole were facing toward the magnet 79 times, and away 11 times (88% towards). Crabs in front of the cardboard "dummy magnet" were facing towards it 154 times and away 28 times (85% towards). Of the crabs behind the magnets, those behind the N pole were facing towards it 64 times, and away 24 times (73% towards), those behind the S pole were facing towards it 58 times and away 30 times (66% towards), and those behind the cardboard were facing towards it 94 times, and away 51 times (65% towards). The differences in total numbers of readings are due to varying numbers of readings of orientation parallel to the magnets, and the presence of

TABLE I

Growth of regenerating limb buds of fiddler crabs exposed to magnetic fields. N = north pole, S = south pole, C = control. In "significance" column, inner value is for difference from controls, or difference of S from N if there was no control (experiments 5, 10–12). Outer numbers indicate difference of S from N, or SS from NN, as indicated by the brackets.

Expt.	Month	Carapace width	# Legs off	Days of growth	Pole	(n)	R value	Signif. by *t* test
2	Dec.	18 mm	2	39	N	(6)	0 +	
					C	(12)	2.4 ± 0.5 (s.e.)	
					S	(6)	8.0 ± 0.8	0.001
3	Feb.	15 mm	6	11	N	(12)	0.8 ± 0.2 (s.e.)	0.001
					C	(12)	2.4 ± 0.3	
					S	(12)	5.2 ± 0.4	0.001
5	May	18 mm	7	10	N	(6)	5.2 ± 1.1 (s.e.)	
					S	(6)	8.4 ± 0.9	0.05
6	June	18 mm	7	12	N	(8)	4.1 ± 0.9 (s.e.)	n.s.
					C	(11)	6.0 ± 0.9	>0.01
					S	(8)	8.3 ± 1.0	0.05
7	June	17 mm	6	14	NN	(6)	4.7 ± 1.5 (s.e.)	0.05
					N	(12)	6.3 ± 1.7	n.s.
					C	(10)	9.6 ± 1.4	>0.01 >0.05
					S	(12)	13.6 ± 1.4	0.05
					SS	(6)	11.1 ± 1.6	n.s.
					NS	(6)	6.8 ± 1.3	n.s.
8	July	17 mm	6	10	NN	(6)	7.7 ± 1.3 (s.e.)	n.s.
					N	(12)	6.4 ± 0.9	0.05
					C	(12)	9.9 ± 0.7	>0.01
					S	(12)	9.7 ± 0.6	n.s.
					SS	(6)	10.1 ± 0.4	n.s.
					NS	(6)	11.3 ± 1.5	n.s.
9	Aug.	15 mm	6	13	NN	(6)	12.5 ± 1.4 (s.e.)	0.05
					N	(12)	13.2 ± 1.3	n.s.
					C	(12)	16.8 ± 1.0	
					S	(12)	16.6 ± 1.2	n.s.
					SS	(6)	17.0 ± 1.0	n.s.
					NS	(6)	15.6 ± 1.5	n.s.
10	Apr.	11 mm	6	9	N	(12)	4.1 ± 0.6 (s.e.)	
					S	(12)	4.5 ± 0.7	n.s.
11	July	11 mm	6	7	N	(16)	5.6 ± 0.9 (s.e.)	
					S	(16)	7.0 ± 1.0	n.s.
12	Aug.	11 mm	6	6	N	(16)	5.1 ± 0.8 (s.e.)	
					S	(16)	4.9 ± 0.5	n.s.
13	July '80	18 mm	3	10	N	(10)	5.2 ± 0.5 (s.e.)	n.s.
					C	(12)	7.2 ± 1.1	
					S	(11)	6.6 ± 0.6	n.s.

twice as many crabs by the cardboard. Of the parallel readings, no differences were seen in numbers of times spent facing left or right in any group. In all groups, crabs spent most of the time facing towards the magnet.

DISCUSSION

In experiments 1–7 proximity to the S pole was correlated with accelerated growth of regenerating limbs, and proximity to the N pole was correlated with retarded growth.

A number of possible mechanisms may be involved in effects of magnetic fields on living systems. Haberditzl (1967) studied enzyme activities and found that carboxydismutase, glutamate dehydrogenase, and trypsin in solution produced gradients in response to a non-uniform magnetic field. Similarly, Barnothy (1964a) has stated that external magnetic fields can change the motion of electrolytes in cells, leading to aggregations of chemical substances at variance with their normal distribution. Neurath (1969) found that development of frog eggs was impaired if they were placed in a strong inhomogeneous field with their animal/vegetal gradient parallel to the field and gradient direction. Presumably, this disrupted the polarity of the eggs, which is necessary for proper development.

Liboff (1965) has stated that magnetic fields interfere with diffusion of ions, and Labes (1966) has shown that a magnetic field of 1000 Oersteds influenced charge transport, both ionic and electronic, as well as reaction rates in biological systems. Gross (1964b) felt that the inhibition of biochemical reactions caused by magnetic fields was due to interference with rotation of paramagnetic molecules, such as free radicals, which can be intermediates in biological processes, and to alterations in bond angle orientation, which would impair closeness of fit of enzymes and substrates. Gualtierotti and Capraro (1964) found that magnetic fields changed the potential of frog skin by decreasing the influx of sodium ions. Since cell growth is related to diffusion of substances across the plasma membrane, these chemical fluxes in response to magnetic fields may be associated with the growth effects.

One can imagine that as a crab moves through a magnetic field, voltage is induced across the limb stump and currents are produced. However, the animal need not move to generate a current. Barnothy and Barnothy (1974) have found that conduction currents are produced in inhomogeneous fields in which different parts of the experimental specimen are exposed to different field strengths. These currents can effect the nervous system. Becker (1961) found that transverse D.C. voltages were obtained when a steady state magnetic field of 2500 Oersteds was applied vertically to a non-moving salamander limb, but only when nerves were intact. When the magnetic field was removed, the original baseline voltage was resumed. Oberg (1973) has found magnetic effects on nerve activity in frogs, and Russell (1969) found that magnetic fields affected nerve impulses in cockroaches.

Bioelectricity has been found to play an important role in regeneration. Borgens *et al.* (1977a) have reported that currents normally leave the tip of regenerating salamander limb buds. Furthermore, the same investigators (1977b) found that the application of a 0.2 μA current to the limb stumps of adults frogs (*Rana pipiens*) could stimulate partial regeneration if the current was cathodal (distally negative). The application of anodal current (distally positive) caused extensive destruction of the limb stump. Control frogs (sham treated and untreated) merely healed. These effects of oppositely directed current may be related to the effects of N and S poles observed here. Borgens *et al.* (1979) found that causing a steady small current to leave limb stumps of *Xenopus* enhanced limb regeneration in this anuran. In both *Rana* and *Xenopus* the cathodally stimulated regenerates had increased the innervation to the regenerating tissue, and the authors felt that the current's effects were mediated by early enhancement of nerve growth. That electric currents can affect crab limb regeneration was demonstrated by Mantel and Levin (1973).

The regenerating limb buds of *Uca* are pointed in a generally upward angle, which is the orientation of the merus. If somehow the crabs by the S pole had currents induced upward (and therefore out of the limb stump) and those by the N pole had current induced downward, this could help explain the effects.

A problem with these possible mechanisms is that the crabs were not immobilized to be subjected to a magnetic field of constant direction, but were free to face in any direction. However, it has been observed that various organisms orient in magnetic fields (Brown *et al.*, 1960a, Brown *et al.*, 1960b, Barnwell and Brown, 1964). Experiments of Gross (1964a) and Gross and Smith (1964) produced results (in retarding tumor growth and wound healing) in unrestrained mice in a horizontal magnetic field. Barnothy (1964b) says that unrestrained animals in a horizontal magnetic field will show attenuation rather than anullment of magnetic effects, since animals will not have opposite orientations for the same length of time, and therefore will not totally cancel out cumulative reversible effects. The preferred orientation of crabs, facing towards the magnets, supports this idea. Gerencser *et al.* (1964) found that inhomogeneous fields reduced growth of bacteria due to a paramagnetic phenomenon. Rod-shaped bacteria were more affected than spherical ones. They suggested that the effects did not cancel out because, although the bacteria were free to change their position, they could have been oriented by the magnetic field and therefore tended to keep their position relative to the field and the gradient. Recently, Frankel *et al.* (1979) found that some bacteria do, in fact, orient in a magnetic field due to the presence of iron as magnetite within them. Magnetite has also been found in the abdomens of bees (Gould *et al.*, 1978), and in the heads of pigeons (Walcott *et al.*, 1979), which also can orient in magnetic fields. Visalberghi and Alleva (1979) found that magnets placed N-pole-up on the heads of homing pigeons caused disorientation. This was not observed in birds with magnets placed S pole up. These studies show that magnetic fields can be perceived by these animals, and can play a role in their normal activities. It is therefore possible that the earth's magnetic field might have adaptive effects on fiddler crabs' daily activities.

Otoliths can be highly paramagnetic and can be influenced by magnetic fields (Barnothy, 1964b). However, the data on orientation of crabs showed that they oriented toward the piece of cardboard as well as toward the magnets. This indicates that the presence of the object, rather than its magnetic field, was responsible for the orientation in these experiments. It is possible, however, that a more extensive and rigorous investigation of orientation would have revealed differences between orientation to the cardboard and to the magnets.

Animals placed in a vertical magnetic field can be unrestrained and still remain in a constant position relative to the field and gradient vectors, since they do not become supine. In our experiments with vertical fields on adult crabs (5 and 6), clear effects were seen. Furthermore, these crabs were extremely active, much more so than any crabs in horizontal fields or in no applied magnetic field. Their hyperactivity may indicate perception of the field and an attempt to orient themselves differently (vertically ?) within it, which they were unable to do.

An asymmetry in the experiments as they were set up is as follows: crabs at both sides of the magnet are exposed to a magnetic field of the same absolute spatial direction (*i.e.* N → S). However, the crabs at the N pole of the magnet are exposed to an inhomogeneous field whose gradient is in the opposite direction, since the gradient vector points in the direction in which increase in field strength occurs. These crabs had reduced growth. Conversely, the crabs at the S pole are experiencing an inhomogeneous field whose gradient vector is in the same direction as the field vector, due to their placement immediately north of the magnet's S pole. Some phenomena have been shown to be gradient sensitive rather than field sensitive (Barnothy, 1964a). These include arrest of tumor growth and lethal effects in *Drosophila* and mice. Barnothy (1964b) has said that an inhomogeneous field exerts an acceleratory force in the direction of the gradient vector on molecules that have an intrinsic magnetic moment, or whose susceptibility differs from that of the environment. This force is independent of the field vector, but depends only on the gradient vector. Our observed effects may be dependent on this property of the inhomogeneous field.

The lack of effects on juveniles and on adults in summer is puzzling. It may be related to the fact that regeneration is normally very rapid during this time, as the crabs approach their normal time of ecdysis, and that they tend to be more refractory to environmental influences. Crabs also have been found to have reduced sensitivity to heavy metal toxicants during this time of year (Weis, 1976b). Thorp and Lake (1974) found a similar seasonal change in the sensitivity of shrimp (*Paratya tasmaniensis*) to cadmium. Juvenile crabs normally go through their molt cycle more rapidly, and their growth has been found to be more refractory to other environmental influences as well (Rao, 1965). It would appear that individuals with more rapid growth rates are less sensitive to environmental effects. However, Fingerman and Fingerman (1979) found that individuals with faster growing limb buds were more sensitive to Aroclor 1242 than those with slowly growing limb buds. Therefore, the reasons for the lack of sensitivity of juveniles and of adults in the summer are not clear. It is possible that juveniles would respond to magnetic fields in the winter months, or if fewer limbs were removed. It is also possible that since the volume of limb buds is less and less tissue is being affected, no significant differences could be observed.

We do not expect that the preceding discussion has thoroughly explained the phenomenon we have observed. Whether or not we can explain it completely, the effects noted are of interest and should be investigated further.

ACKNOWLEDGMENTS

We wish to thank Drs. V. Santarelli, B. Sonnenblick, S. McDowell, and P. Weis for reviewing the manuscript and offering their comments. Ms. Jennifer Weis is thanked for her technical assistance. This research was supported by Biomedical Research Support Grant RR7059. P.H.L. was a student in the Honors Program during the course of this research.

LITERATURE CITED

BARNOTHY, J. M., 1963. Growth rate in mice in static magnetic fields. *Nature*, **200**: 86–87.

BARNOTHY, J. M., 1964a. Introduction. Pp. 3–24 in M. Barnothy, Ed. *Biological Effects of Magnetic Fields*. Vol. I. Plenum Press, New York.

BARNOTHY, J. M., 1964b. The vector character of field and gradient and its possible implications for biomagnetic experiments and space travel. Pp. 56–62 in M. Barnothy, Ed. *Biological Effects of Magnetic Fields*. Vol. I. Plenum Press, New York.

BARNOTHY, J. M., M. F. BARNOTHY, AND I. BOSZORMENYI-NAGY, 1956. Influence of a magnetic field upon the leukocytes of the mouse. *Nature*, **177**: 577–578.

BARNOTHY, M. F., AND J. M. BARNOTHY, 1974. Magnetobiology. Pp. 313–349 in N. B. Slonim, Ed. *Environmental Physiology*. C. B. Mosby Co., St. Louis.

BARNWELL, F. H., AND F. A. BROWN, JR., 1964. Responses of planarians and snails. Pages 263–278 in M. F. Barnothy, Ed. *Biological Effects of Magnetic Fields*. Vol. I. Plenum Press, New York.

BECKER, R. O., 1961. Search for evidence of axial current flow in peripheral nerves of the salamander. *Science*, **134**: 101–102.

BLISS, D. E., 1956. Neurosecretion and the control of growth in a decapod crustacean. Pp. 56–75 in K. G. Wingstrand, Ed. *Bertil Hanstrom, Zoological Papers in Honor of his Sixty-fifth Birthday, Nov. 20, 1956*. Zool. Inst. Lund, Sweden.

BLISS, D. E., 1960. Autotomy and regeneration. Pp. 561–589 in T. Waterman, Ed. *Physiology of Crustacea*. Academic Press, New York.

BLISS, D. E., AND J. R. BOYER, 1964. Environmental regulation of growth in the decapod crustacean *Gecarcinus lateralis*. *Gen. Comp. Endocrinol.* **4**: 15–41.

BOE, A. A., AND D. K. SALUNKHE, 1963. Effects of magnetic fields on tomato ripening. *Nature,* **199**: 91–92.

BORGENS, R., J. VANABLE, AND L. JAFFE, 1977a. Bioelectricity and regeneration: Large currents leave the stumps of regenerating newt limbs. *Proc. Nat. Acad. Sci. U. S. A.,* **74**: 4528–4532.

BORGENS, R., J. VANABLE, AND L. JAFFE, 1977b. Bioelectricity and regeneration I. Initiation of frog limb regeneration by minute currents. *J. Exp. Zool.,* **200**: 403–416.

BORGENS, R., J. VANABLE, AND L. JAFFE, 1979. Small artificial currents enhance *Xenopus* limb regeneration. *J. Exp. Zool.,* **207**: 217–226.

BROWN, F. A., JR., M. F. BENNETT, AND H. M. WEBB, 1960a. A magnetic compass response of an organism. *Biol. Bull.,* **119**: 65–74.

BROWN, F. A., JR., W. J. BRETT, M. F. BENNETT, AND F. H. BARNWELL, 1960b. Magnetic response of an organism and its solar relationships. *Biol. Bull.,* **118**: 367–381.

COOK, E. S., J. C. FARDON, AND L. G. NUTINI, 1969. Effect of magnetic fields on cellular respiration. Pp. 67–78 in M. F. Barnothy, Ed. *Biological Effects of Magnetic Fields.* Vol. 2. Plenum Press, New York.

EISELEIN, B. S., H. M. BOUTELL, AND M. W. BIGGS, 1961. Biological effects of magnetic fields—negative results. *Aerosp. Med.,* **32**: 383–386.

FINGERMAN, S., AND M. FINGERMAN, 1979. Effects of two polychlorinated biphenyls (Aroclors 1242 and 1254) on limb regeneration in the fiddler crab, *Uca pugilator,* at different times of the year. *Vie Milieu,* **28–29**: 69–75.

FRANKEL, R. B., R. P. BLAKEMORE, AND R. S. WOLFE, 1979. Magnetite in freshwater magnetotactic bacteria. *Science,* **203**: 1355–1356.

GERENCSER, V. F., M. F. BARNOTHY, AND J. M. BARNOTHY, 1964. Inhibition of bacterial growth in fields of high paramagnetic strength. Pp. 229–239 in M. F. Barnothy, Ed. *Biological Effects of Magnetic Fields.* Vol. 1. Plenum Press, New York.

GOULD, J. L., J. L. KIRSCHVINK, AND K. S. DEFFEYES, 1978. Bees have magnetic remanence. *Science,* **201**: 1026–1028.

GROSS, L., 1964a. Lifespan increase of tumor-bearing mice through pretreatment. Pp. 132–139 in M. F. Barnothy, Ed. *Biological Effects of Magnetic Fields.* Vol. 1. Plenum Press, New York.

GROSS, L., 1964b. Distortion of the bond angle in a magnetic field and its possible magnetobiological implications. Pp. 74–79 in M. F. Barnothy, Ed. *Biological Effects of Magnetic Fields.* Vol. 1. Plenum Press, New York.

GROSS, L., AND L. W. SMITH, 1964. Wound healing and tissue regeneration. Pp. 140–145 in M. F. Barnothy, Ed. *Biological Effects of Magnetic Fields.* Vol. 1. Plenum Press, New York.

GUALTIEROTTI, T., AND V. CAPRARO, 1964. The action of magnetic field on sodium transport across cell membrane. Pp. 311–316 in M. Florkin and A. Dollfus, Eds. *Life Sciences and Space Research. A Symposium.* John Wiley & Sons, New York.

HABERDITZL, W., 1967. Enzyme activity in high magnetic fields. *Nature,* **213**: 72–73.

LABES, M. M., 1966. A possible explanation for the effect of magnetic fields on biological systems. *Nature,* **211**: 968.

LIBOFF, R. L., 1965. A biomagnetic hypothesis. *Biophys. J.,* **5**: 845–853.

MALININ, G. I., W. D. GREGORY, L. MORELLI, V. K. SHARMA, AND J. C. HOUCK, 1976. Evidence of morphological and physiological transformation of mammalian cells by strong magnetic fields. *Science,* **194**: 844–846.

MANTEL, L. H., AND R. S. LEVIN, 1973. Effects of multiple limb removal and stimulation on regeneration and molting in *Uca pugilator*. *Am. Zool.,* **13**: 1339.

MULAY, I. L., AND L. W. MULAY, 1961. Effect of a magnetic field on Sarcoma 37 ascites tumor cells. *Nature,* **190**: 1019.

NEURATH, P. W., 1969. The effect of high gradient, high strength magnetic fields on the early embryonic development of frogs. Pp. 177–187 in M. F. Barnothy, Ed., *Biological Effects of Magnetic Fields.* Vol. 2. Plenum Press, New York.

OBERG, P. A., 1973. Magnetic stimulation of nerve tissue. *Med. Biol. Eng.,* **11**: 55–64.

RAO, K. R., 1965. Studies on the influence of environmental factors on growth in the crab *Ocypode macrocera* H. Milne Edwards. *Crustaceana,* **11**: 257–276.

RUSSELL, D. R., 1969. Effect of a constant magnetic field on invertebrate neurons. Pp. 227–232 in M. F. Barnothy, Ed., *Biological Effects of Magnetic Fields.* Vol. 2. Plenum Press, New York.

RENO, V. R., AND L. G. NUTINI, 1963. Effect of magnetic fields on tissue respiration. *Nature,* **198**: 204–205.

SKINNER, D. M., AND D. E. GRAHAM, 1972. Loss of limbs as a stimulus to ecdysis in Brachyura (true crabs). *Biol. Bull.,* **143**: 222–233.

TANEEVA, A. I., 1978. Changes in physiological processes in hydrobionts under the effect of a constant magnetic field. *Gidrobiol. Zh.,* **114**: 56–63.

THORP, V. J., AND P. S. LAKE, 1974. Toxicity bioassays of cadmium on selected freshwater invertebrates and the interaction of cadmium and zinc on the freshwater shrimp, *Paratya tasmaniensis* (Riek). *Austr. J. Mar. Freshw. Res.,* **25**: 97–104.

VISALBERGHI, E., AND E. ALLEVA, 1979. Magnetic influences on pigeon homing. *Biol. Bull.,* **156**: 246–256.

WALCOTT, C., J. L. GOULD, AND J. L. KIRSCHVINK, 1979. Pigeons have magnets. *Science,* **205**: 1027–1028.

WEIS, J. S., 1976a. Effects of environmental factors on regeneration and molting in fiddler crabs. *Biol. Bull.,* **150**: 52–62.

WEISS, J. S., 1976b. Effects of mercury, cadmium, and lead salts on regeneration and ecdysis in the fiddler crab, *Uca pugilator.* *Fish. Bull.,* **74**: 464–467.

WEIS, J. S., 1977. Limb regeneration in fiddler crabs: species differences and effects of methylmercury. *Biol. Bull.,* **152**: 236–274.

Reference: *Biol. Bull.*, **159**: 692–699. (December, 1980)

RESPIRATORY METABOLISM OF *MACROBRACHIUM OLFERSII* (WIEGMANN) ZOEAE DURING THE MOULTING CYCLE FROM ECLOSION TO FIRST ECDYSIS

JOHN C. McNAMARA,[1] GLORIA S. MOREIRA, AND PLINIO S. MOREIRA

Instituto Oceanográfico and Instituto de Biociências, Universidade de São Paulo, Brasil

ABSTRACT

The respiratory metabolism of first stage *Macrobrachium olfersii* (Wiegmann) zoeae was measured at 12 hr intervals with Cartesian diver microrespirometers throughout the 120 hr period from eclosion to first ecdysis. Weight-specific oxygen consumption rates, although slightly higher immediately after hatching, remained constant and did not differ significantly during the first moulting cycle. Larval dry weight decreased from 21.8 to 18.9 μg during this period. There was no evidence of a diurnal metabolic pattern. The moulting cycle was subdivided according to morphological characteristics, based on the degree of cytoplasmic homogeneity, epidermal retraction, and stage of setal development in the telson. Larval metabolism is suggested to be uniform throughout the eclosion–first ecdysis period, in spite of a distinct and divisible moulting cycle.

INTRODUCTION

Although some aspects of adult crustaceans' physiology have been well studied in relation to the moulting cycle (see Passano, 1960; Charniaux-Cotton and Kleinholz, 1964; Drach and Tchernigovtzeff, 1967; Novales *et al.*, 1973; and Kleinholz, 1976 for references), the relationships between respiratory rate and moult-inhibiting hormone, and the putative control of respiration by a separate eyestalk hormone, still are not well understood (Silverthorn, 1975a, b; Kleinholz, 1976). Several authors have studied the respiratory metabolism of intact adult crustaceans as a function of the moulting cycle (Scudamore, 1947; Roberts, 1957; Costlow and Bookhout, 1958; Halcrow and Boyd, 1967; Bulnheim, 1974; Hagerman, 1976). However, such studies of the relationship between larval metabolism and moulting are lacking.

The moulting cycle of some larval decapod crustaceans has been subdivided and morphologically characterized (Rao *et al.*, 1973; Van Herp and Bellon-Humbert, 1978; Huner and Colvin, 1979; Freeman and Costlow, 1980), although palaemonid larvae have received little attention. The present study evaluates the possible influence of moulting-cycle-related events on the respiratory metabolism of the first zoea of a palaemonid shrimp, *Macrobrachium olfersii* (Wiegmann), and offers a tentative morphological basis for the subdivision of the moulting cycle of early zoeae of this species.

Received March 25, 1980; accepted September 25, 1980.

[1] Address for reprint requests: Departamento de Fisiologia, Instituto de Biociências, Caixa Postal 11.461, Universidade de São Paulo, 05421 São Paulo, Brasil.

MATERIALS AND METHODS

Ovigerous females of the freshwater palaemonid shrimp *Macrobrachium olfersii* were maintained in the laboratory in aerated aquaria filled with river water (5‰ salinity). Specimens were collected from the lower reaches of the Guaecá River (approx. 23° 49′ 18″ S; 45° 27′ 18″ W) in the State of São Paulo, Brazil. Larvae, usually released in the early evening between 2000 and 2200 hr, were collected by pipette, transferred to small glass bowls containing 100 ml of river water mixed with seawater to a final salinity of 14‰, and kept in a constant temperature chamber at 20°C under 12 hr light: 12 hr dark. The larvae were not fed throughout the 5-day experimental period, since our previous results showed larval yolk reserves to be sufficient nourishment during the first zoeal stage (Moreira *et al.*, 1979).

Oxygen consumption of individual Stage I zoeae was measured using Cartesian diver microrespirometers (Holter, 1943), each having a total volume of between 8 and 13 μl. Measurements were made at 12 hr intervals beginning 2 hr after hatching, for 120 hr, which in this species effectively represents the first larval moulting cycle. The respiratory rates of a minimum of seven larvae were determined at each 12 hr interval. Zoeae were micropipetted into 0.8 μl of dilute seawater, 14‰ salinity. Following an equilibration period of 30 min, diver readings were taken at 30 min intervals over 1 hr, during which values were constant. Such measurements represent "routine metabolism" (Prosser, 1973), since zoeae could move slightly, but not swim, inside the divers. All experiments were performed at 20°C (controlled by a thermostat regulating with 0.01°C). In order to render the equilibrium pressure independent of barometric changes, one end of the manometer was connected by a thick-walled capillary tube to a 4-1 barostatic bottle completely submerged in a water bath.

Each zoea was used only once and was examined after removal from the respirometer to verify condition and degree of activity. Results are expressed both as μl O_2 consumed·mg dry $wt^{-1}\cdot hr^{-1}$ and μl O_2 consumed·$larva^{-1}\cdot hr^{-1}$. Significant differences of means were tested according to Parl (1967).

To determine successive larval dry weights, 15 larvae from selected batches (at 24, 72, and 96 hr) were briefly rinsed with distilled water, dried overnight at 80°C, placed in a dessicator for 2 hr and weighed on a Cahn Gram electrobalance (0.1 μg sensitivity).

Groups of larvae, after being used for respirometric determinations, were preserved separately in 4% formalin. The tails of these larvae were severed at the junction of the telson and last abdominal segment, mounted in glycerine, and covered with a coverslip. Photomicrographs of the inner 4th and 5th telson setae were taken with Kodak Panatomic 32 ASA film using an Olympus photomicroscope.

RESULTS

Figure 1 shows the respiratory rates of newly hatched Stage I *Macrobrachium olfersii* zoeae, measured at 12 hr intervals over a 120 hr period. Larval dry weights were calculated as 21.8, 19.9, and 18.9 μg at 24, 72, and 96 hr, respectively, after hatching. The small weight loss was presumably due to the larvae's use of yolk reserves. Weight-specific oxygen-consumption rates were slightly higher immediately after hatching (2 hr) but quickly reached a stable level after 26 hr. No significant difference in respiratory rate was recorded during the subsequent experimental period, including possible alterations associated with measurements made

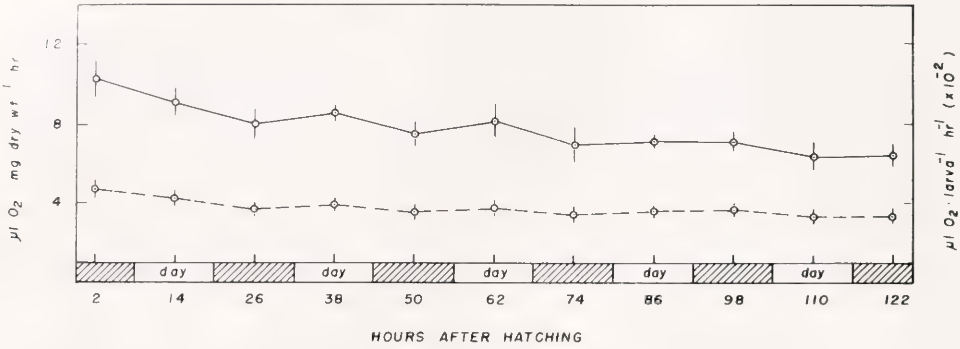


FIGURE 1. Rate of oxygen consumption by State I *Macrobrachium olfersii* zoeae during 120 hrs from eclosion to first ecdysis. Solid line, $\mu l O_2$ consumed · larva^{-1} · hour^{-1}; broken line, $\mu l O_2$ consumed · milligram dry weight^{-1} · hour^{-1}. Vertical bars = SE of the mean.

during daylight and night hours. Weight-specific oxygen-consumption rates appeared to be somewhat lower just before moulting, but differences were not statistically significant. One zoea actually moulted inside the diver with no modification in respiratory rate recorded.

Oxygen consumption per animal steadily diminished over the experimental period, although no significant differences were recorded after 26 hr post-hatching, in spite of the concomitant decrease in larval dry weight.

During the 120 hr experimental period most larvae underwent a complete moulting cycle (Figs. 2–7). During the first 26 hr after hatching, larvae pass from Stage A to Stage B. In Stage A (Fig. 2) the epidermal and setal cytoplasm is dense, with cellular elements clearly evident. The epidermis completely fills the spaces under the setae and their articulations. In Stage B (Fig. 3) the epidermal cytoplasm becomes more homogenous, still filling the space under the cuticle. Cone-like structures begin to form at the setal bases. After 50 hr, the larvae have reached substage D_0 (Fig. 4) in which the epidermis has begun to retract from the cuticle between the setae. The setal cytoplasm, however, remains clearly connected to the telson epidermis. By 74 hr, most larva have attained at least substage D_1' (Fig. 5), characterized by a well defined epidermal retraction between the setae and at their bases. The partially formed new seta is surrounded by a bulb-like invagination, the proximal end of which is not well defined in the epidermal matrix. After 96 hours, most larvae have reached substage D_1''' (Fig. 6). Setal invagination is complete, although the open ends of the new setae are not well defined. Barbules can be seen on the new setal walls. New setules are evident in the space between the retracted epidermis and the cuticle. Immediately after the moult to the second zoea (betwen 96 and 120 hr), setal and epidermal components (Fig. 7) appear identical to those seen in Stage A of the newly hatched zoea I (Fig. 2).

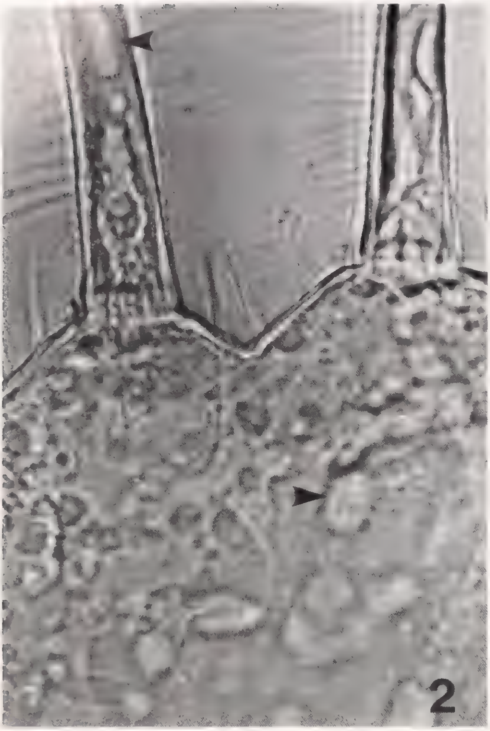
DISCUSSION

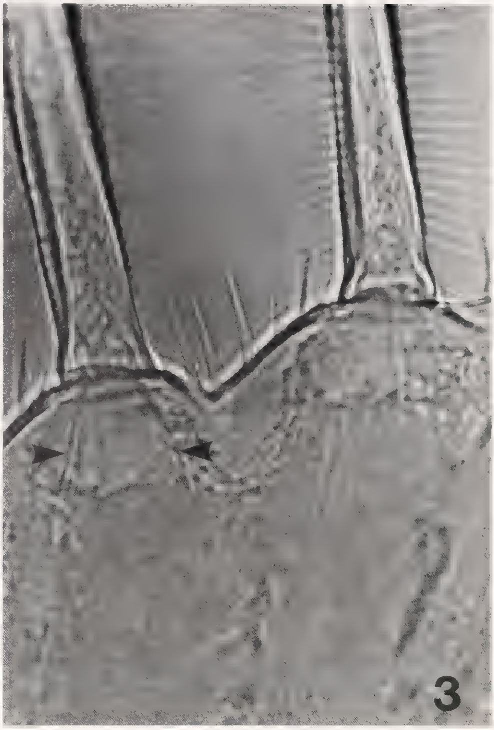
Scudamore (1947), Scheer and Scheer (1954), Roberts (1957), Halcrow and Boyd (1967), Paranjape (1967), Bulnheim (1972, 1974), and Hagerman (1976) all reported increased oxygen consumption associated with the immediate pre- and/or post-moult periods in a variety of intact adult crustaceans, including amphipods, decapods, euphausiids, and isopods. Skinner (1962) has also shown that oxygen consumption by isolated integumentary tissues increases during exocuticle synthesis, immediately prior to ecdysis in *Gecarcinus lateralis.* However, some researchers have reported results similar to those of the present study, *e.g.*, Costlow and Bookhout (1958) and Barnes and Barnes (1963), studying the cirripedes *Balanus amphitrite* and *Balanus balanoides*, respectively, demonstrated there was either no significant increase (former) or only a small increase (latter) in respiratory rate at ecdysis. Vernberg and Costlow (1966) noted a lack of significant difference in respiratory rate for *Uca pugnax* first zoeae during 3 days.

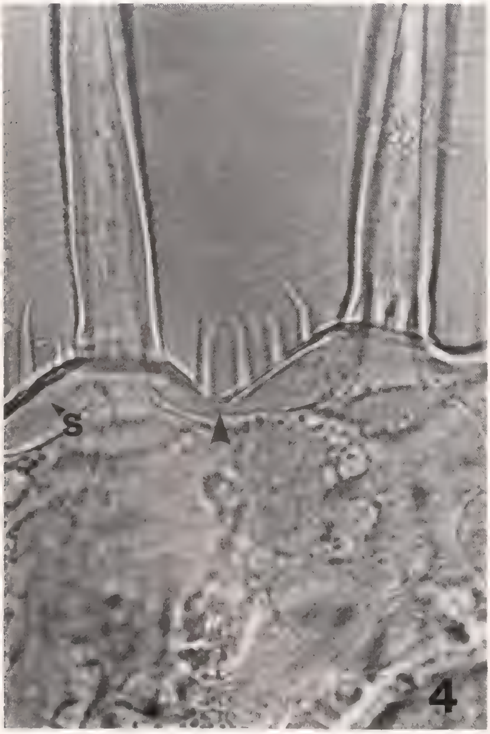
Crustacean metabolism has been postulated to be either directly regulated by specific eyestalk hormones (Silverthorn, 1975a, b) or indirectly modified by moult-related hormones also released from eyestalk neurosecretory centers (Passano, 1960). On this basis, a functional analysis of the neurosecretory structures in the larval crustacean eyestalk is essential to understanding of larval respiratory responses. Hubschman (1963) showed that the sinus gland is not recognizable until the fifth zoeal stage in several species of *Palaemonetes* and that the ganglionic X-organs are not functional during larval development. Significantly, eyestalk removal had no effect on the larval moulting cycle nor on metamorphosis. Bellon-Humbert *et al.* (1978) also showed that the sinus gland in *Palaemon serratus* is not discernable until the fifth zoeal stage, becoming active only after metamorphosis. The ganglionic X-organs (medullae externa and terminalis) are not recognizable before metamorphosis in this species, appearing only during the postlarval phase.

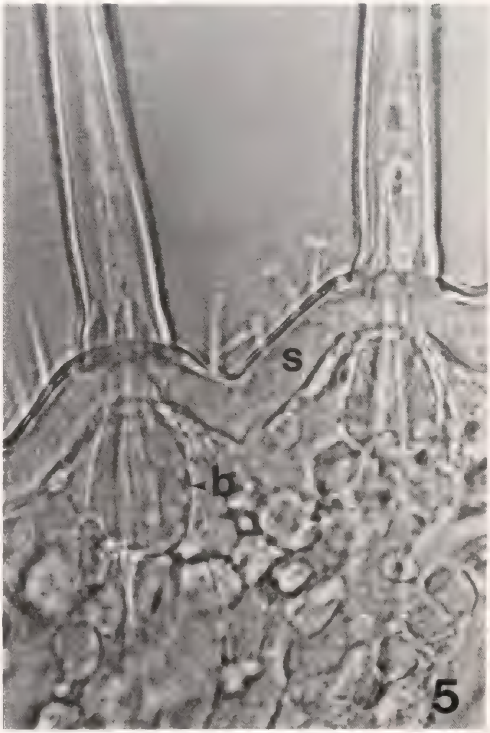
Our findings that moulting-cycle-related events do not affect the respiratory metabolism of *Macrobrachium olfersii* first zoeae, combined with the ontogenetic data of Hubschman (1963) and Bellon-Humbert *et al.* (1978) above, suggest that if a moult-inhibiting hormone is present in early palaemonid zoeae in the eyestalks or elsewhere, it does not appear to affect respiration. Such evidence also indirectly suggests that the larval moulting cycle may be uninhibited, *i.e.,* the larval Y-organ, if it exists and is functional, may produce moult-promoting substances continuously. The rapid moulting cycles (3–4 days) of *Macrobrachium olfersii* and *M. holthusi* early zoeae (Moreira *et al.*, 1979) certainly suggest this. It is unfortunate that so little is known of larval neurosecretory processes in relation to moulting control. Clearly, as suggested by Hubschman (1963), such processes are very different from those known to operate in adults.

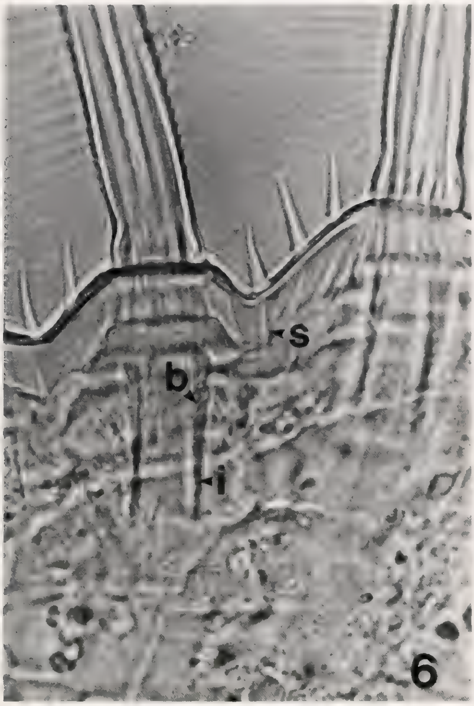
Few authors have studied the morphological changes in epidermal and cuticular structure accompanying larval moulting cycles. Broad and Hubschman (1963), observing the larval development of *Palaemonetes kadiakensis*, noted gross morphological details associated with post-moult stages and suggested that the proecdysial stages may be of relatively long duration. Rao *et al.* (1973) briefly characterized Stages C and D_0-D_1''' of fourth and fifth stage *Homarus americanus* larvae. Like the first zoea of *M. olfersii*, Stage D_0 in these larvae was characterized by retraction of the epidermis, and Stages D_1'-D_1''' by formation of new setae. However, contrary to the situation in *M. olfersii* first zoeae, the new setae are completely separated from the old cuticle and setae. Van Herp and Bellon-Humbert (1978) have produced the most complete subdivision of the larval moulting cycle to date, using second–fourth stage *Astacus leptodactylus* larvae. The characteristics of each stage of the moulting cycle resemble those of *M. olfersii* first zoeae, although the nerve fiber described from *A. leptodactylus* setae was not observed in *M. olfersii* setae. Similarly, the conelike structures described at the setal bases in Stage B and the bulblike structures (setal organ?) described in Stage D_1' of *M. olfersii* larvae were not noted in *A. leptodactylus* larval uropods. Huner and Colvin


2


3


s
4


s
b
5



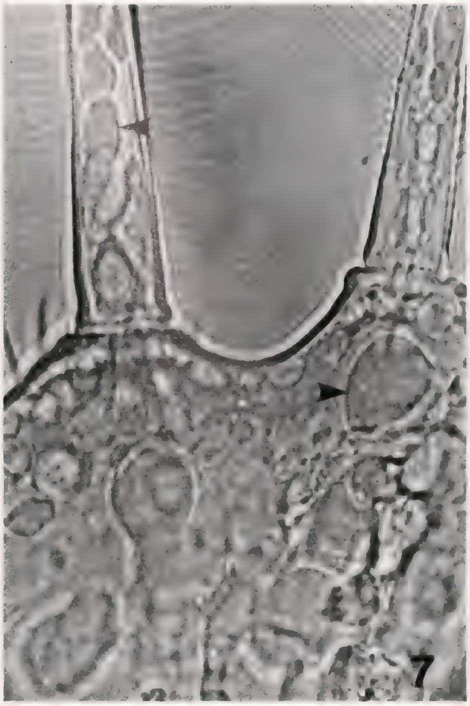


FIGURE 2. Telson and setae of *Macrobrachium olfersii* zoea I in Stage A. Cellular elements (arrows) prominent in both setal and epidermal cytoplasm. 300×.

FIGURE 3. Stage B. Both setal and epidermal cytoplasm is homogeneous. Cone-like structures visible (arrows) at setal bases. 300×.

FIGURE 4. Substage D_0. Epidermis has begun to retract from cuticle leaving a well defined space *s*, narrower in the intersetal areas (arrow). 300×.

FIGURE 5. Substage D_1'. Epidermal retraction *s* closely follows curves of cuticle with uniform spacing. Bulb-like invagination *b* surrounds the base of each forming seta. 300×.

FIGURE 6. Substage D_1'''. Setal invagination *i* is complete. Barbules, *b*, project from internal walls of new seta. Setules, *s*, appear in the space between old cuticle and retracted epidemis. 300×.

FIGURE 7. Telson and setae of *Macrobrachium olfersii* zoea II in Stage A. Cellular elements (arrows) are evident in heterogeneous setal and epidermal cytoplasm. 300×.

(1979) described moulting cycle subdivisions for juvenile *Penaeus californiensis* and *P. stylirostris* that approximate those of *M. olfersii* first zoeae, differing only in some details, *e.g.*, the absence of inner setal cones in Stage C of *Penaeus* juveniles. Freeman and Costlow (1980) characterized the zoeal and megalopal moulting cycles of *Rhithropanopeus harrisii* using the morphological changes occurring in the integument, particularly in the spines and antennae. In *R. harrisii* larvae, the general events correlate with those taking place in *M. olfersii* first zoeae. However, a direct comparison was not possible as these authors did not describe phases of setal development.

Although conforming to the general scheme outlined by Drach and Tchernigovtzeff (1967) for crustacean moulting cycle subdivision, our preliminary data for *M. olfersii* first zoeae have revealed more morphological details than previously described for larval crustaceans. As previously suggested by McNamara (1979), detailed studies at the cellular level are necessary to elucidate the complex modifications occurring in the crustacean integument during the moulting cycle. The first area of *M. olfersii* has proved to be excellent material for such studies owing to its fine transparent cuticle and simple epidermis.

The present study has emphasized that although modifications in metabolic rate are usually associated with the moulting cycle of crustaceans, such modifications do not necessarily occur in early developmental stages, suggesting that studies throughout the developmental sequence are necessary to a better understanding of the relationship between metabolism and the crustacean moulting cycle.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to Dr. L. Ludovico George of the Biomedical Sciences Institute, University of São Paulo, for the use of photomicrographic equipment. This study was supported in part by the Organization of American States (OAS).

LITERATURE CITED

Barnes, H., and M. Barnes, 1963. The relation of water uptake and oxygen consumption of the body tissues to the moulting cycle in *Balanus balanoides* (L.). *Crustaceana,* **6**: 62–68.

Bellon-Humbert, C., M. J. Thijssen, and F. Van Herp, 1978. Development, location and relocation of sensory and neurosecretory sites in the eyestalks during the larval and post-larval life of *Palaemon serratus* (Pennant). *J. Mar. Biol. Assoc. U. K.,* **58**: 851–868.

Broad, A. C., and J. H. Hubschman, 1963. The larval development of *Palaemonetes kadiakensis.* M. J. Rathbun in the laboratory. *Trans. Am. Microsc. Soc.,* **82**: 185–197.

Bulnheim, H.-P., 1972. Vergleichende Untersuchungen zur Atmungsphysiologie euryhaliner Gammariden unter besonderer Berkücksichtigung der Salzgehaltsanpassung. *Helgoländer Wiss. Meeresunters.,* **23**: 485–534.

Bulnheim, H.-P., 1974. Respiratory metabolism of *Idotea balthica* (Crustacea, Isopoda) in relation to environmental variables, acclimation processes and moulting. *Helgoländer Wiss. Meeresunters.,* **26**: 464–480.

Charniaux-Cotton, H., and L. H. Kleinholz, 1964. Hormones in invertebrates other than insects. Pp. 135–198 in G. Pincus, K. V. Thimann, and E. B. Astwood, Eds., *The Hormones.* Academic Press, New York.

Costlow, J. D., Jr., and C. G. Bookhout, 1958. Molting and respiration in *Balanus amphitrite* var. *denticulata* Broch. *Physiol. Zool.,* **31**: 271–280.

Drach, P., and C. Tchernigovtzeff, 1967. Sur la méthode de détermination des stades d'intermue et son application générale aux crustacés. *Vie et Milieu, Ser. A.,* **18**: 595–607.

Freeman, J. A., and J. D. Costlow, 1980. The molt cycle and its hormonal control in *Rhithropanopeus harrisii* larvae. *Develop. Biol.,* **74**: 479–485.

Hagerman, L., 1976. The respiration during the moulting cycle of *Crangon vulgaries* (Fabr.) (Crustacea, Natantia). *Ophelia,* **15**: 15–21.

Halcrow, K., and C. M. Boyd, 1967. The oxygen consumption and swimming activity of the amphipod *Gammarus oceanicus* at different temperatures. *Comp. Biochem. Physiol.,* **23**: 233–242.

Holter, H., 1943. Technique of the Cartesian diver. *C. R. Trav. Lab. Carlsberg. Ser. Chim.,* **24**: 399–478.

Hubschmann, J. H., 1963. Development and function of neurosecretory sites in the eyestalks of larval *Palaemonetes* (Decapoda: Natantia). *Biol. Bull.,* **125**: 96–113.

Huner, J. V., and L. B. Colvin, 1979. Observations on the molt cycle of two species of juvenile shrimp, *Penaeus californiensis* and *Penaeus stylirostris* (Decapoda: Crustacea). *Proc. Nat. Shellfish. Assn.,* **69**: 77–84.

Kleinholz, L., 1976. Crustacean neurosecretory hormones and physiological specificity. *Amer. Zool.,* **16**: 151–166.

McNamara, J. C., 1979. Ultrastructural observations on the endoplasmic reticulum of epidermal cells in the branchiostegite of *Macrobrachium holthuisi* (Crustacea: Decapoda). *Biol. Fisiol. Animal, Univ. S. Paulo,* **3**: 73–80.

Moreira, G. S., J. C. McNamara and P. S. Moreira. 1979. The combined effects of temperature and salinity on the survival and moulting of early zoeae of *Macrobrachium holthuisi* (Decapoda: Palaemonidae). *Biol. Fisiol. Animal, Univ. Sao Paulo,* **3**: 81–93.

Moreira, G. S., J. C. McNamara, P. S. Moreira, and M. Weinrich, 1980. Temperature and salinity effects on the respiratory metabolism of the first zoeal stage of Macrobrachium holthuisi Genofre & Lobão (Decapoda: Palaemonidae). *J. Exp. Mar. Biol. Ecol.,* **47**: in press.

Novales, R. R., L. I. Gilbert, and F. A. Brown, Jr., 1973. Endocrine mechanisms. Pp. 857–908 in C. L. Prosser, Ed., *Comparative Animal Physiology.* W. B. Saunders Co., Philadelphia.

Paranjape, M. A., 1967. Molting and respiration of euphausiids. *J. Fish. Res. Bd. Canada,* **24**: 1229–1240.

Parl, B., 1967. *Basic Statistics.* Doubleday and Co., New York, 364 pp.

Passano, L. M., 1960. Molting and its control. Pp. 473–536 in T. H. Waterman, Ed.. *The Physiology of Crustacea.* Vol. 1, Academic Press, New York.

Prosser, C. L., 1973. Oxygen: respiration and metabolism. Pp. 163–211 in C. L. Prosser, Ed., *Comparative Animal Physiology.* 3rd Edition. W. B. Saunders Co., Philadelphia.

Rao, K. R., S. W. Fingerman, and M. Fingerman, 1973. Effects of exogenous ecdysones on the molt cycles of fourth and fifth stage American lobsters, *Homarus americanus. Comp. Biochem. Physiol.,* **44**: 1105–1120.

Roberts, J. L., 1957. Thermal acclimation of metabolism in the crab *Pachygrapsus crassipes* Randall. I. The influence of body size, starvation and moulting. *Physiol. Zool.,* **30**: 232–242.

Scheer, B. T.. and M. A. Scheer, 1954. The hormonal control of metabolism in crustaceans. VIII. Oxygen consumption in *Leander serratus. Pubbl. Staz. Zool. Napoli,* **25**: 419–426.

Scudamore, H. H., 1947. The influence of the sinus glands upon moulting and associated changes in the crayfish. *Physiol. Zool.* **20**: 187–208.

Silverthorn, S. S., 1975a. Hormonal involvement in thermal acclimation in the fiddler crab *Uca pugilator* (Bosc). I. Effect of eyestalk extracts on whole animal respiration. *Comp. Biochem. Physiol.,* **50**: 281–283.

Silverthorn, S. S., 1975b. Hormonal involvement in thermal acclimation in the fiddler crab Uca pugilator (Bosc). II. Effects of extracts on tissue respiration. *Comp. Biochem. Physiol.,* **50**: 285–290.

Skinner, D. M., 1962. The structure and metabolism of a crustacean integumentary tissue during a molt cycle. *Biol. Bull.,* **123**: 635–647.

Van Herp, F., and C. Bellon-Humbert, 1978. Setal development and molt prediction in the larvae and adults of the crayfish. *Astacus leptodactylus* (Nordman, 1842). *Aquaculture,* **14**: 289–301.

Vernberg, F. J., and J. D. Costlow, Jr., 1966. Studies on the variation between tropical and temperature-zone fiddler crabs of the genus *Uca.* IV. Oxygen consumption of larvae and young crabs reared in the laboratory. *Physiol. Zool.,* **39**: 36–52.

Vernberg, W. B., G. S. Moreira, and J. C. McNamara, 1980. The effect of temperature on the respiratory metabolism of the developmental stages of *Pagurus criniticornis* (Dana) (Anomura: Paguridae). *Mar. Biol. Letters,* in press.

Reference: *Biol. Bull.*, **159**: 700–713. (December, 1980)

ON THE FORM AND SIZE OF CRAYFISH LEGS REGENERATED AFTER GRAFTING

JAY EDWARD MITTENTHAL

Department of Biological Sciences, Purdue University, W. Lafayette, Indiana 47907

ABSTRACT

The control of form and size in regenerated legs of crayfish was investigated by interchanging basipodites of the cheliped and first walking leg on one side. Homotypic, homoeotic, and mosaic legs regenerated. A C(cheliped)-like or W(walking-leg)-like leg usually regenerated with normal proportions, independent of size and sex of the animal and of host site.

These factors affected the type and size of a leg. The dimensions of the propodite increased allometrically with body length and were larger for chelipeds of males than of females. The C site promoted more rapid growth than the W site. Legs adjacent to a regenerating leg apparently influenced its type and size: C-type tissue growing at either host site tended to inhibit the regeneration of C-type tissue, and to decrease the growth rate of either type of leg, at the adjacent site.

In Crustacea a limb bud may be partitioned into exopodite and endopodite compartments. Atypical development in an exopodite compartment may underlie the observed regeneration of atypical motile exopodites. The majority of mosaic walking legs have the anterior face W-like and the posterior face C-like; the characteristic boundary between these faces may separate anterior and posterior compartments in the endopodite.

INTRODUCTION

Serially homologous structures in a segmented animal can be used to analyze pattern formation during development. The five pairs of legs (pereiopods) of decapod crustaceans show deviations from strict serial homology and, in some species, from bilateral symmetry. This variety of similar but distinctive forms, the capacity for regeneration of legs, and the relatively large size of the animals invite the study of pattern formation through surgical perturbation (reviewed by Needham, 1965).

Although the relationships between the time course of regeneration and the molt cycle in crustaceans have been intensively investigated (*e.g.*, Bliss, 1960; Durand, 1960), the available information about development or regeneration in crustacean legs is largely descriptive (*e.g.*, Emmel, 1910).

In this study, regeneration after interchange of a proximal segment between stumps of two crayfish legs showed that the host site can influence the size and pos-

---

Received June 15, 1979; accepted September 25, 1980.

Abbreviations: C: cheliped (first pereiopod); W: walking leg (second pereiopod); C-like C: cheliped-like leg regenerated at site normal for C; W-like W: W-like leg regenerated at site normal for W; W-like C: W-like leg regenerated at site normal for C; C-like W: C-like leg regenerated at site normal for W; homotypic leg: leg of type normally at the host site (*e.g.*, C-like C, W-like W); homoeotic leg: leg of type normally at a different host site (*e.g.*, W-like C, C-like W).

[1] Present address: Department of Biology, University of Oregon, Eugene, Oregon 97403.

sibly the type of regenerated leg. Host sites and adjacent legs seemed to interact in determining the size and type of each regenerated leg. Each type of leg usually had proportions independent of its size and host site.

Materials and Methods

Animals and maintenance

Juvenile specimens of the crayfish *Procambarus clarkii* were obtained from Monterey Bay Hydroculture Farms, Santa Cruz, California, or raised from eggs. Animals were maintained individually after surgery in circulating filtered well water at 18°–22°C. They were fed various combinations of carrots, beef or chicken liver, Tetramin fish food, and a pelletized crayfish food obtained from the supplier. The light : dark cycle was approximately 10 hr light : 14 hr darkness.

Grafting

Basal segments of the anterior two pairs of legs, the cheliped (C) and the first walking leg (W; Fig. 1), were interchanged on one side of each animal. Operations were performed at all phases of the molt cycle (Stevenson, 1968) later than 2 days after a molt. Animals often died when chilled soon after molting. Grafts transplanted 1 or 2 days before a molt were often lost in molting.

The operation was as follows: A crayfish of 20–30 mm body length (tip of rostrum to caudal margin of telson) was anesthetized by gradual chilling to 0°–5°C in well water. The animal was confined ventral side up with tungsten staples in a groove cut into a slab of Sylgard (Dow-Corning) in a petri dish. The animal was immersed at 0°–5°C in the saline of van Harreveld (1936), pH 7.0, with 10 mM Tris replacing bicarbonate buffer. A C or W was broken off at the basipodite-ischiopodite joint by crushing the meropodite or twisting the ischiopodite ventralward (*cf.* Wales *et al.*, 1970). It is unclear whether this discarding of distal segments is a reflex (autotomy) or a fracture at a region of weakness (autospasy, Wood and Wood, 1932), particularly in the walking legs. On the C and W the coxopodite-basipodite joint was then transected with iridectomy scissors and the tip of a fine syringe needle (26–28 gauge). Each basipodite was pressed into the host coxopodite in normal orientation. After the operation the animal was returned to well water at 0°C and warmed slowly to room temperature.

Early in the study the above procedure was not performed in a single operation. Rather, the C and W were broken off, and then the animal was maintained until after the next molt occurred. Each basipodite was then transplanted with its regenerating limb bud. For later animals fracture and interchange were combined in one operation. Both procedures yielded similar legs, which are described here.

As a "remove-and-replace" control series, basipodites of the C and W on one side were ablated and then pressed into the coxopodites of the donor legs. A second control series is described in "Results."

Muscle in the coxopodite and transplanted basipodite was often opaque a few days after transplantation. Such opacity is presumably a sign of cell death in and around the graft, since healthy muscle is translucent. A transplanted limb bud projecting from the coxopodite was usually opaque. It was often unclear whether a grafted basipodite had been retained after a scab and opacity obscured the interior of the coxopodite.

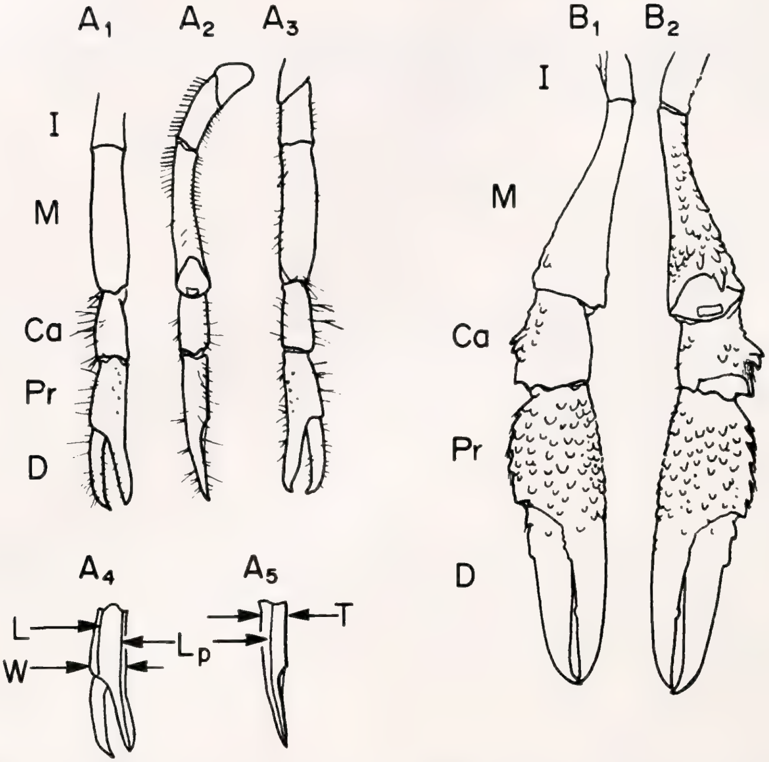


FIGURE 1. Cheliped and first walking leg of *Procambarus*. Leg segments: ischiopodite (I), meropodite (M), carpopodite (Ca), propodite (Pr), dactylopodite (D). The terms anterior, posterior, dorsal, and ventral refer to the position of structures when the leg is fully extended perpendicular to the antero-posterior axis of the body. A: Normal walking leg, posterior (A_1, A_4), ventral (A_2, A_5), and anterior (A_3) views. No tubercles are present; arcs on the propodite represent small pits. The rows of large setae are dorsal (left in A_1) and antero-ventral (right in A_2, left in A_3). A second row of smaller setae occurs on the ventral meropodite posterior to the first; it is sometimes more prominent than in this animal. A_4, A_5: Parameters used to characterize size and shape of propodites. L_p: propodite length; L: manus length; W: manus width; T: manus thickness. The measurement of L_p from the ventral view was used only when the index curved markedly in this view. B: Normal cheliped. Because of torsion at the Ca-Pr joint the views corresponding to those for the walking leg are, meropodite: B_1: dorsal, B_2: ventral; propodite: B_1: posterior, B_2: anterior. Note the two somewhat irregular rows of large tubercles on the ventral meropodite (B_2) and the rows of tubercles on both faces of the propodite.

Form and size of legs

Legs were observed on animals of body length 55–80 mm. These animals had molted at least 3–9 times after the interchange operation, 3–13 months earlier. Dimensions of propodites were measured on drawings of the legs made at 3X–12X with a Wild M5 microscope with drawing attachment.

RESULTS

Types of regenerates

At the site of the cheliped and walking leg regenerated legs were cheliped-like, walking leg-like, or mosaic. Discrete markers allowed recognition of C-like and W-like regions in mosaic legs (Fig. 1).

Mosaic legs

The majority (15/27) of the mosaic legs at the W site had the anterior half W-like and the posterior half C-like (Fig. 2). The division between W-like and C-like halves was especially clear on the ventral surface of the meropodite, where an anterior row of W-like setae paralleled a posterior row of C-like tubercles of variable prominence. Occasionally (2/27 animals), one or two tubercles appeared in the anterior row. A few mosaic walking legs appeared W-like except that tubercles replaced setae in the posterior row.

In mosaic W with differing anterior and posterior portions (antero-posterior), the posterior surfaces of the meropodite and carpopodite, which are smooth in normal W, often had low tubercles. The carpopodite was abnormally broad but had W-like anterior rows of setae.

Tubercles occur on a normal C, and setae on a normal W, on the propodite and dactylopodite. The propodites of antero-posterior mosaic W had setae on the anterior face and tubercles on the posterior face. The tubercles differed in promi-

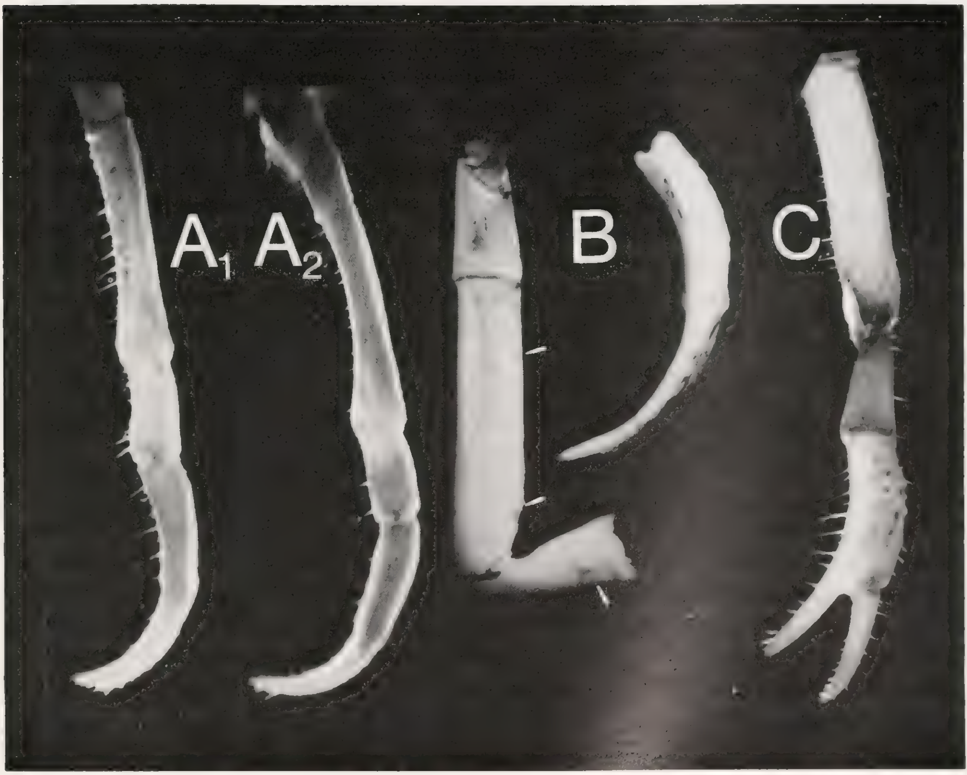


FIGURE 2. Antero-posterior mosaic walking legs. All four legs shown are from right side of animal. A_1, A_2, and C are backlighted to show setae, so tubercles appear as dark spots in these pictures. A_1, A_2: Typical antero-posterior mosaic W, ventral view. Rows of W-like setae occur on the anterior side of the meropodite and propodite (left side of picture), and rows of C-like tubercles on the posterior side. Curvature of the index toward the anterior side is greater than in a walking leg. B, C: Variant mosaic W. B: Anterior view of proximal segments, ventral view of propodite. The leg resembles those shown in A_1 and A_2 except for a few tubercles on the anterior side of the meropodite and carpopodite (arrows). C: posterior view. Postero-dorsal aspect is C-like, ventral and anterior aspects W-like.

range, orderliness of rows, and dorsoventral distribution among animals, but were always restricted to the posterior half of the leg.

In the antero-posterior mosaic W the index and dactylopodite often curved more than normally toward the anterior. Posterior C-type cells may proliferate faster than anterior W-type cells to increase distal curvature. The curvature of index and dactylopodite sometimes differed, with larger tubercles proximal to the member with greater curvature.

In six mosaic W not of antero-posterior type, numerous tubercles appeared in the anterior half of one or more leg segments. Five of the six legs had two ventral rows of tubercles, and the sixth had anterodorsal tubercles, on the meropodite. On other segments tubercles were restricted to a part of the circumference, differing among legs. Patches containing tubercles occurred only in one segment in some legs, but extended across joints in others.

No type of mosaic C predominated. The seven mosaic C examined spanned the spectrum for mosaic W. Two of the four mosaic C with differing anterior and posterior halves were C-like anteriorly and W-like posteriorly. The index and dactylopodite of these mosaic C curved toward the posterior face, presumably because the anterior C-type surface grew more rapidly. The other two antero-posterior mosaic C approximated large antero-posterior mosaic W, but the boundary between C-like and W-like regions was more variable.

Two control series of operations were performed to investigate why mosaic legs regenerate. In the "remove-and-replace" series, 3 of 23 animals regenerated antero-posterior mosaic W; the remaining regenerates appeared normal for the site. Thus the operation used in the experimental series to interchange basipodites can induce mosaic regeneration; the interchange of basipodites between C and W sites is not essential.

Damage to a basipodite during surgery might make it more vulnerable to influence from the host site, increasing the probability that a mosaic or host-like leg would regenerate. Specifically, inadvertent damage to the anterior proximal margin of the cheliped basipodite might favor regeneration of an antero-posterior mosaic W. To test this possibility, in a second control series the proximal 30% of homolateral C and W basipodites was ablated with a cut parallel to the distal margin of the basipodite. This cut removed both anterior and posterior parts of the proximal margin. The residues of the basipodites were then interchanged. The W site regenerated a mosaic leg in 15 of 18 crayfish; 10 of these legs were antero-posterior mosaic W. Thus damage to the C basipodite often allowed the W site to influence the regenerated leg. However, regeneration of an antero-posterior mosaic W does not require preferential damage to the anterior proximal margin of the C basipodite.

Exopodites

A small fraction of the regenerated legs were biramous, unlike typical uniramous C and W. These atypical legs had an exopodite attached to the dorsal part of the basipodite at the joint with the ischiopodite (Fig. 3). Each exopodite consisted of a larger proximal segment and a series of smaller distal segments bearing setae.

Like the exopodites of the maxillipeds, the atypical exopodites beat intermittently. Exopodites of pereiopods and maxillipeds usually did not beat in synchrony. However, both seemed to beat at roughly the same frequency—about 1–2 beats/sec at 3°C in a 70 mm male. One animal regenerated exopodites on a mosaic C and on

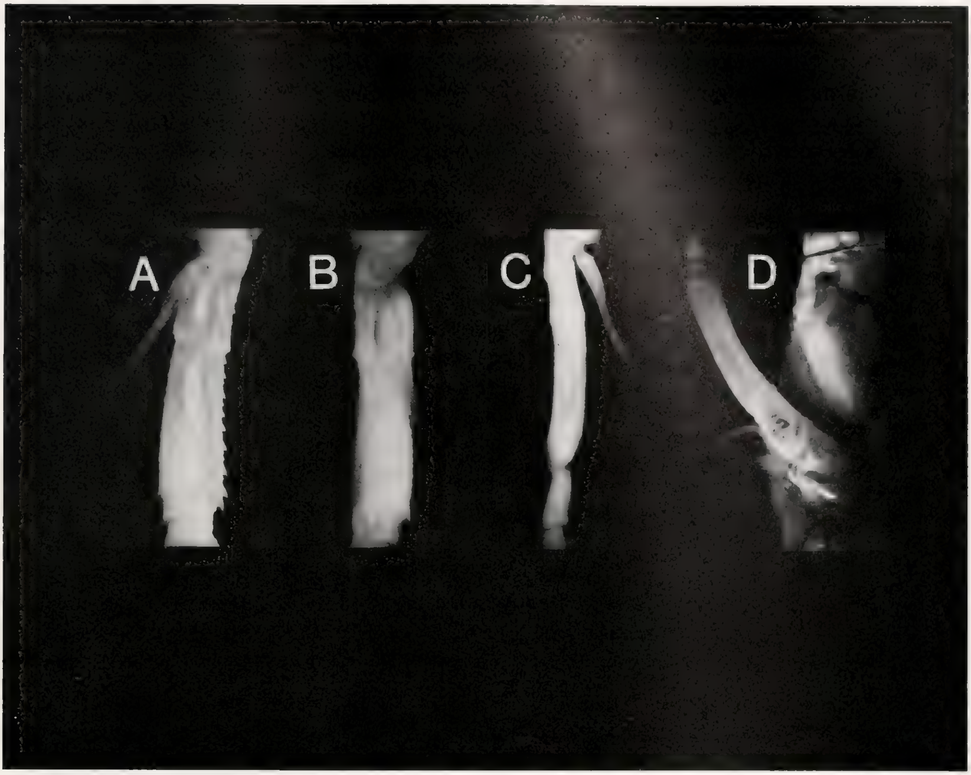


FIGURE 3. Exopodites of regenerated periopods. A: cheliped; B: mosaic cheliped; C, D: walking leg-like cheliped. A, B are anterior views; C, D are posterior views. In D the tip of the expodite is blurred because the exopodite was beating.

the adjacent C-like W; these exopodites could beat independently of each other and of the exopodites of the maxillipeds.

Exopodites were also observed on two W-like C's. Regeneration of an exopodite does not occur only on homeotic or mosaic legs, however. In the "remove-and-replace" control series, 2 of 23 animals regenerated exopodites on C-like C. Surgery is not necessary, and regeneration of a leg may not be necessary, to produce a post-embryonic exopodite: A cheliped having a motile exopodite occurred in one unoperated 29 mm male. Examination of the most immature animals available, four preserved normal specimens of *Procambarus* in the second stage after hatching, showed no exopodites on the pereiopods.

Motility of regenerated legs

Most regenerated legs were used in the manner typical for an unoperated leg at the host site. A crayfish walking on a level substrate held a leg at the C site off the substrate, while a leg at the W site touched the substrate periodically in the stepping rhythm. (One exceptional animal held a C-like W off the substrate during walking. This leg was lost at the next molt, and neither C site nor W site subsequently regenerated a leg.) Typically the regenerated legs showed normal closing and defense reflexes involving the dactylopodite. These observations suggest

TABLE I

Ratios of dimensions of propodites; mean + s.d. (number of legs). "Control" legs are from unoperated animals measured as obtained from the supplier. "Normal" legs are unoperated legs from operated animals. Manus length ranged from 2.2 to 15.2 mm in chelipeds, and from 2.7 to 6.7 mm in walking legs, used to estimate these ratios.

	Propodite length / Manus length	Manus width / Manus length	Manus thickness / Manus length
Control C	2.63 ± 0.21 (13)	0.79 ± 0.11 (13)	0.56 ± 0.07 (12)
Normal C	2.54 ± 0.18 (21)	0.81 ± 0.10 (21)	0.55 ± 0.06 (20)
C-like C	2.61 ± 0.22 (9)	0.76 ± 0.09 (9)	0.51 ± 0.08 (9)
C-like W	2.73 ± 0.10 (3)	0.76 ± 0.03 (3)	0.47 ± 0.01 (3)
Control W	2.04 ± 0.08 (11)	0.48 ± 0.05 (11)	0.33 ± 0.03 (10)
Normal W	2.13 ± 0.11 (24)	0.53 ± 0.05 (25)	0.34 ± 0.03 (23)
W-like W	2.14 ± 0.18 (6)	0.53 ± 0.05 (6)	0.34 ± 0.02 (6)
W-like C	2.00 ± 0.21 (10)	0.48 ± 0.06 (10)	0.35 ± 0.04 (9)

that the regenerated leg usually made functionally adequate neural connections with the ganglion at the host site.

Size of the legs

Propodite length, manus length, manus width, and manus thickness (Fig. 1) were used to compare normal and regenerated legs. Sixteen unoperated animals and 21 animals bearing legs regenerated after grafting were measured. C-like legs or W-like legs maintained constant proportions, regardless of site or time of growth (Table I; but note thickness/length for manus of C-like legs). C-like and W-like legs showed distinct patterns of proportional growth; the propodite length, manus width, and manus thickness increased more rapidly, relative to manus length, in C-like than in W-like legs. Although walking legs of unoperated males and females grew at the same rate, sexual dimorphism occurred in chelipeds: In unoperated animals the propodite of the cheliped was about 1.25 times as long in males as in females (Fig. 4).

C-like, W-like, and mosaic regenerates were larger at the C site than at the W site (Table II). The regenerated legs were intermediate in size between the contralateral unoperated C and W.

Frequency distribution of types of regenerates

At the C site and at the W site three types of legs regenerated: C-like, W-like, and mosaic. Thus nine pairings of types might have regenerated at the two sites. Table III summarizes the prevalence of the types, singly and in pairs, in 47 animals. At both sites the majority of regenerated legs were not of graft type, but were host-like or mosaic. Also, at both sites regeneration of heteromorphic legs was biased toward smaller, less specialized types—at the C site, W-like C rather than mosaic C; at the W site, mosaic W rather than C-like W.

Three of the nine pairings of leg types occurred in about two-thirds of the animals. In the most common pairing (15/47), the two more common types of heteromorphic legs, W-like C and mosaic W, occurred together. In 16/47 animals a C-like C accompanied a mosaic W or W-like W. Of the remaining six pairings

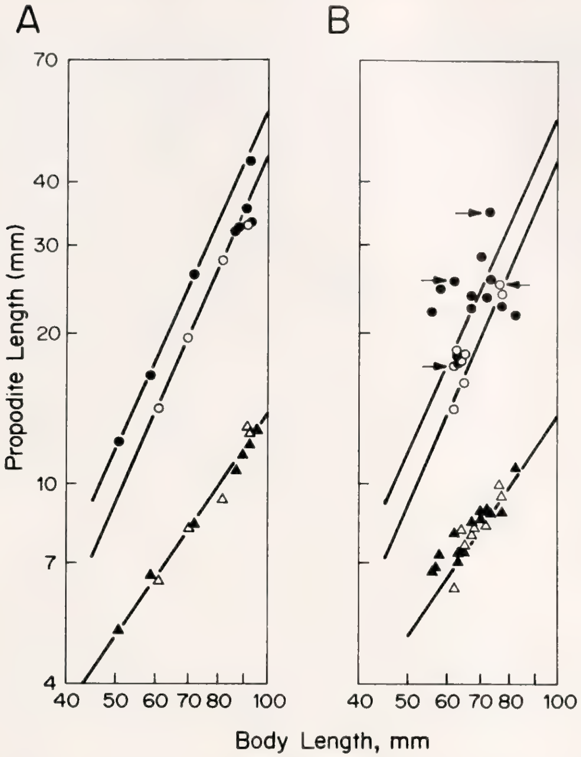


FIGURE 4. Propodite length vs. body length. Circle, chelipid; triangle, walking leg; blackened symbol, male; open symbol, female. A. Unoperated animals, measured as obtained from the supplier. Lines are drawn by eye. B. Normal C and W of operated animals. Lines are from part A, for unoperated animals. Arrows indicate legs for which mass of closer muscle was measured; see Mittenthal *et al.*, this volume.

of leg types, the most common was W-like C with C-like W. This pairing regenerated in less than 10% of the animals.

Table III includes estimates of the number of animals expected to bear each of the nine pairings if legs regenerated independently at the C site and W site. Of the six pairings in which the two regenerated legs were of different types, four occurred more often than expected from independent regeneration. All three of the pairings having both legs of the same type occurred less often than expected. The probability that the two legs regenerated independently is less than 0.16.

As might be expected from the prevalence of mosaic W, legs at the C site were paired with mosaic W at least as often with W-like W or C-like W. It was therefore interesting to ask whether each of the three types of regenerates at the C site was preferentially paired with antero-posterior mosaic W or with other mosaic W. Table IV shows that the larger was the type of leg regenerated at the C site, the greater the probability of its being paired with an antero-posterior mosaic W.

DISCUSSION

In these experiments homolateral basipodites of the cheliped and first walking leg of crayfish were interchanged. If each graft had retained its initial state of

TABLE II

For each of four indices of propodite dimensions, the ratio of the index in a regenerated limb to the index in a contralateral normal limb is given (mean + s.d.). Each set of four ratios was compared to the reference set (1, 1, 1, 1) with the single-sample T^2 statistic (Morrison, 1967). The last column gives the probability P *that the regenerated limb is not significantly different from the control limb; * = insufficient data for test.*

Type of limb		No. of animals	Ratio, regenerated/control				*P*
Regenerated limb	Control limb		Propodite length	Manus length	Manus width	Manus thickness	
W-like W	normal W	6	1.05 ± 0.06	1.03 ± 0.04	1.08 ± 0.05	1.09 ± 0.05	>0.1
W-like C	normal W	8	1.16 ± 0.12	1.19 ± 0.15	1.13 ± 0.14	1.21 ± 0.20	>0.1
Mosaic W	normal W	11	1.30 ± 0.16	1.17 ± 0.12	1.31 ± 0.16	1.35 ± 0.17	<0.005
Mosaic W	normal C	11	0.49 ± 0.09	0.54 ± 0.08	0.37 ± 0.07	0.37 ± 0.07	<0.005
Mosaic C	normal W	3	1.48 ± 0.11	1.44 ± 0.31	1.56 ± 0.28	2.01 ± 0.41	*
Mosaic C	normal C	3	0.58 ± 0.16	0.63 ± 0.23	0.47 ± 0.06	0.58 ± 0.06	*
C-like W	normal C	1	0.90	0.88	0.82	0.65	*
C-like C	normal C	7	0.94 ± 0.07	0.93 ± 0.12	0.84 ± 0.08	0.83 ± 0.09	<0.1

TABLE III

Number of animals with various types of regenerated legs in cheliped and walking leg coxopodites. For each entry, the number on the left is observed. The number on the right, in parentheses, is calculated on the hypothesis that the types of legs regenerated in cheliped and walking leg coxopodites are independent. The probability that the number of mosaic C and W-like C is ≤ 6 *is 0.154 on this hypothesis, by Fisher's exact test (Blalock, 1960). For this test the entries of Table I were pooled by using leg types C-like C, W-like W, other C, other W.*

In cheliped coxopodite In walking leg coxopodite	C-like C	Mosaic C	W-like C	Number of animals with given type leg in walking leg coxopodite
W-like W	7 (4.98)	3 (1.94)	3 (6.09)	13
Mosaic W	9 (10.34)	3 (4.02)	15 (12.64)	27
C-like W	2 (2.68)	1 (1.04)	4 (3.28)	7
Number of animals with given type leg in cheliped coxopodite	18	7	22	47

determination, and if no grafts were lost, each operated animal would have regenerated two homoeotic legs, a walking leg-like cheliped and a cheliped-like walking leg. However, in addition to homoeotic legs, host-type and mosaic legs regenerated. Similarly, Edwards and Sahota (1967) found homoeotic, mosaic, and host-type regenerates in crickets after they grafted the bud of a regenerating cercus to the stump of a mesothoracic leg. Mosaic appendages may have originated from the grafts through a combination of cell death, invasion of the graft by host tissue, and transdetermination of graft tissue. These processes or loss of grafts could have produced host-type regenerates.

The results of this experiment show that the proportions of the leg are independent of its size and are determined with its type (*cf.* Prange, 1977).

However, other distinctive correlates of leg type, the surface features of the cuticle, do change with size in decapod Crustacea. As a crayfish grows, the number and prominence of tubercles on the cheliped increase. Factor (1978) has shown that the distribution of tubercles and setae on the perioral appendages of the lobster *Homarus* changes during the molts between larval and postlarval stages.

The host site for regeneration of a leg affects its growth rate; the cheliped site promotes more rapid growth than the walking leg cite. The use of a leg may also affect its growth rate. The normal cheliped of operated animals tended to be

TABLE IV

Number of animals having two types of mosaic W paired with various types of limbs at the C site. A-p = antero-posterior. For this table, mosaic W with any tubercles on the anterior face were included in "not a-p."

Type of leg at C site Type of mosaic W	C-like C	Mosaic C	W-like C
a-p	6	2	6
not a-p	1	1	5

larger than a cheliped of an unoperated animal of the same size. The normal cheliped may be used more, and may hypertrophy, after the operation.

In decapod crustaceans a cheliped can influence the determination of the contralateral cheliped. The *Alpheidae* (snapping shrimp) have asymmetric chelipeds, a large specialized snapper and a smaller, less specialized pincer. After autotomy of the snapper, the pincer is transformed to a snapper, and a pincer replaces the lost snapper (Przibram, 1931; Darby, 1934). To explain reversal of asymmetry, it has been postulated that the snapper suppresses the transformation of the pincer to a snapper (Wilson, 1903; Darby, 1934; Mellon and Stephens, 1978).

The present results suggest that in crayfish the cheliped and first walking leg are analogous to the snapper and the pincer, respectively, in interactions between legs. Cheliped-type (C-type) epidermis in either leg apparently tends to prevent regeneration of C-type tissue in the other leg. Pairing of a walking leg-like (W-like) regenerate at one host site and a C-type or mosaic regenerate at the other site was prevalent; pairing of two C-like legs was rare.

C-like tissue may also decrease the growth rate of legs at adjacent sites. (See Huxley, 1972, for an alternative hypothesis on growth modulation.) A regenerated C-like C tends to be smaller than the contralateral normal C. However, a W-like W tends to be larger than the contralateral unoperated W, perhaps because a small C-site regenerate exerts subnormal inhibition on growth of the W-site regenerate. This hypothesis predicts a greater enlargement of the W-site regenerate, the less C-like is the regenerated leg at the C site, as was observed.

If C-type tissue reduces growth of adjacent legs, then contralateral unoperated legs, growing under reduced inhibition from smaller regenerating legs, should tend to be larger than homologous legs of unoperated animals. This is so: A propodite of an unoperated C or W tends to be longer in an operated animal than in a comparable unoperated animal (Figure 4B), especially for male chelipeds.

Thus in *Procambarus* chelipeds may inhibit the growth of adjacent legs. C-type tissue in a cheliped or mosaic leg may suppress formation of C-type tissue in adjacent legs. It remains to be seen whether suppression of growth and of C-type determination are causally related.

The majority of mosaic walking legs were W-like on the anterior half, adjacent to the C site, and C-like on the posterior half. The prevalence of this pattern suggests that the proposed suppression of C-type development by adjacent C-type tissue is spatially graded, decreasing with distance from the C-type tissue. Polarization of a mosaic W toward this pattern should then be more frequent, the more C-like is the regenerate at the C site. The results in Table IV agree with this prediction.

In the antero-posterior mosaic W the boundary between W-type and C-type epidermis is particularly clear on the ventral surface of the meropodite. There an anterior row of W-like setae parallels a posterior row of C-like tubercles. It is difficult to believe that graded inhibition from the C site falls below a threshold for developing C-like tissue exactly between the rows of protuberances in most mosaic W.

The phenomenon of compartmentalization in *Drosophila* offers a model for interpreting a characteristic W-C boundary in an antero-posterior mosaic W. In *Drosophila* determination of an imaginal disc proceeds through a sequence of compartmentalization events (Crick and Lawrence, 1975; García-Bellido, 1975). In each event boundaries are established at characteristic positions in the disc, delimiting new compartments. The progeny of cells within a compartment do not cross its boundaries, although cells may cross boundary lines not yet established. Mutants are known that change the state of determination in particular compartments, apparently leaving the rest of the disc unaltered. During regeneration of a disc, boundaries previously set can be crossed by clones, and compartmentalization proceeds anew as the disc develops (Szabad *et al.*, 1979).

The *Minute* (M) mutant has been used to demonstrate compartmentalization. X-irradiation of a *Drosophila* larva heterozygous for *Minute* may induce formation of an M^+/M^+ cell by somatic crossing-over. This cell proliferates faster than surrounding M/M^+ cells. The M^+/M^+ patch, revealed by genetic markers, often grows fast enough to fill most of a compartment, so that the boundaries of patch and compartment coincide extensively (Morata and Ripoll, 1975).

Partition of a developing leg into anterior and posterior compartments may occur in crayfish, as in *Drosophila* (Tokunaga, 1962; Steiner, 1976). The compartment boundary might be visible in antero-posterior mosaic W because a patch of C-type cells in the posterior compartment grew faster than surrounding W-type cells and so occupied most of the compartment. Anterior and posterior compartments may also occur in appendages of cockroaches (French, 1976) and crickets (Edwards and Sahota, 1967).

The segments of a crayfish leg distal to the basipodite represent one ramus, the endopodite, of an appendage that was biramous during ontogeny. Normally astacurans shed the other ramus, the exopodite, of each pereiopod at the molt from the last mysis stage to the first post-larval stage (Anderson, 1973). In the present study most regenerated legs lacked an exopodite; some, however, had one. The observation that an appendage of *Procambarus,* or of *Asellus* (Needham, 1950), may regenerate either in uniramous or biramous form suggests that the two rami develop from separate compartments. Presence or absence of a ramus could then correspond to two interpretations of the positional information in a compartment. Atavistic regeneration, in which a phylogenetically earlier type of ramus replaces a lost ramus (*e.g.* Schultz, 1905; reviewed by Needham, 1965), could represent yet another interpretation of the same positional information.

It may seem remarkable that a motile appendage can regenerate after the normal appendage of the same type was lost several molts previously. However, Gurney (1942, pp. 106–107) mentions several decapods in which an appendage is lost at one stage of development but later reappears in functional form. In some Crustacea (Bittner, 1973) axotomy of a mature motoneuron often does not kill the neuron, but initiates changes that culminate in regeneration of its axon. Davis and Davis (1973) have found that in *Homarus* the motoneurons innervating pleopod muscles appear to develop normally, at least to the sixth post-hatching stage, after their target muscles are destroyed at hatching. Perhaps the motoneurons innervating exopodites of pereiopods also survive the normal loss of their target muscles for several stages and can reinnervate a regenerated exopodite.

ACKNOWLEDGMENTS

I thank Dr. Arthur Winfree and Ms. Vickery Trinkaus-Randall for a critical reading of an early draft of the manuscript, and Ms. Glenna D. Cummings for aid with photography. This research was supported in part by NSF grant BNS 77-24149.

LITERATURE CITED

Anderson, D. T., 1973. *Embryology and Phylogeny in Annelids and Arthropods.* Pergamon Press, New York, xiv, 495 pp.

Bittner, G. D., 1973. Degeneration and regeneration in crustacean neuromuscular systems. *Am. Zool.,* **13**: 379–408.

Blalock, H. M., Jr., 1960. *Social Statistics.* McGraw-Hill Book Co., Inc., New York, xiv, 465 pp.

Bliss, D. E., 1960. Autotomy and regeneration. Pp. 561–589 in T. H. Waterman, Ed., *Physiology of Crustacea.* Academic Press, New York, Vol. I.

Crick, F. H. C., and P. A. Lawrence, 1975. Compartments and polyclones in insect development. *Science,* **189**: 340–347.

Darby, H. H., 1934. The mechanism of asymmetry in the *Alpheidae. Carnegie Inst. Wash. Publ.,* **435**: 357–361.

Davis, W. J., and K. B. Davis, 1973. Ontogeny of a simple locomotor system: Role of the periphery in the development of central nervous circuitry. *Am. Zool.,* **13**: 409–425.

Durand, J. B., 1960. Limb regeneration and endocrine activity in the crayfish. *Biol. Bull.,* **118**: 250–261.

Edwards, J. S., and T. S. Sahota, 1967. Regeneration of a sensory system: The formation of central connections by normal and transplanted cerci of the house cricket *Acheta domesticus. J. Exp. Zool.,* **166**: 387–396.

Emmel, V. E., 1910. A study of the differentiation of tissues in the regenerating crustacean limb. *Am. J. Anat.* **10**: 109–159.

Factor, J. R., 1978. Morphology of the mouthparts of larval lobsters, *Homarus americanus* (Decapoda: Nephropidae), with special emphasis on their setae. *Biol. Bull.,* **154**: 383–408.

French, V., 1976. Leg regeneration in cockroach, *Blatella germanica.* II. Regeneration from a noncongruent tibial graft-host junction. *J. Embryol. Exp. Morphol.,* **35**: 267–301.

García-Bellido, A., 1975. Genetic control of wing disc development in *Drosophila.* Pp. 161–182 in *Cell Patterning* (CIBA Foundation Symposium 29, New series). Associated Scientific Publishers, New York.

Gurney, R., 1942. *Larvae of decapod Crustacea.* Ray Society, London, vi, 306 pp.

Huxley, J., 1972. *Problems of Relative Growth.* Second Edition, Dover Publications, Inc., New York, xxvii, 312 pp.

Mellon, D., Jr., and P. J. Stephens, 1978. Limb morphology and function are transformed by contralateral nerve section in snapping shrimps. *Nature,* **272**: 246–248.

Morata, G., and P. Ripoll, 1975. Minutes: Mutants of *Drosophila* autonomously affecting cell division rate. *Dev. Biol.,* **42**: 211–221.

Morrison, D. F., 1967. *Multivariate Statistical Methods.* McGraw-Hill Book Co., New York, xiii, 338 pp.

Needham, A. E., 1950. Determination of the form of regenerating limbs in *Asellus aquaticus. Q. J. Microsc. Sci.,* **91**: 401–418.

Needham, A. E., 1965. Regeneration in the arthropods and its endocrine control. Pp. 283–323 in V. Kiortsis and H. A. L. Trampusch, Eds., *Regeneration of Animals and Related Problems.* North Holland Publishing Co., Amsterdam.

Prange, H. D., 1977. The scaling and mechanics of arthropod exoskeletons. Pp. 169–181 in T. J. Pedley, Ed., *Scale Effects in Animal Locomotion.* Academic Press, New York, xx, 545 pp.

Przibram, H., 1931. *Connecting Laws in Animal Morphology.* University of London Press, Ltd., London, 62 pp.

Schultz, E., 1905. Ueber atavistische regeneration bei Flusskrebsen. *Wilhelm Roux' Arch. Entwichlungsmech. Org.,* **20**: 38–47.

Steiner, E., 1976. Establishment of compartments in developing leg imaginal discs of *Drosophila melanogaster. Wilhelm Roux' Arch. Entwichlungsmech. Org.* **180**: 9–30.

Stevenson, J. R., 1968. Metecdysial molt staging and changes in the cuticle in the crayfish *Orconectes sanborni* (Faxon). *Crustaceana,* **14**: 169–177.

Szabad, J., P. Simpson, and R. Nothiger, 1979. Regeneration and compartments in *Drosophila. J. Embryol. Exp. Morph.,* **49**: 229–241.

Tokunaga, C., 1962. Cell lineage and differentiation on the male foreleg of *Drosophila melanogaster. Dev. Biol.* **4**: 489–516.

van Harreveld, A., 1936. A physiological solution for fresh-water crustaceans. *Proc. Soc. Exp. Biol. and Med.,* **34**: 428–432.

WALES, W., F. CLARAC, M. DANDO, AND M. LAVERACK, 1970. Innervation of the receptors present at the various joints of the pereiopods and third maxilliped of *Homarus gammarus* (L.) and other macruran decapods (Crustacea). *Z. Vergl. Physiol.*, **68**: 345–384.

WILSON, E. B., 1903. Notes on the reversal of asymmetry in the regeneration of the chelae in *Alpheus heterochelis*. *Biol. Bull.*, **4**: 197–210.

WOOD, F. D., AND H. E. WOOD, II., 1932. Autotomy in decapod crustacea. *J. Exp. Zool.* **62**: 1–55.

Reference: *Biol. Bull.*, **159**: 714–727. (December, 1980)

MORPHOLOGY OF THE CLOSER MUSCLES IN NORMAL AND HOMOEOTIC LEGS OF CRAYFISH[1]

JAY EDWARD MITTENTHAL,[2] MARY C. OLSON, AND
GLENNA D. CUMMINGS

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

ABSTRACT

Regeneration of homoeotic legs in crayfish was evoked by grafting a proximal leg segment to an ectopic host site, in order to investigate the relative roles of the host site and the donor leg type in determining morphological characteristics of a leg muscle, the dactylopodite closer. To provide a frame of reference for results from closers of homoeotic chelipeds and first walking legs, closers from the corresponding legs of unoperated animals were also examined.

The mass, fiber length, and fiber diameter of normal and regenerated closers increase in proportion to appropriate external dimensions of a leg: Mass is proportional to a measure of the volume of the manus, where the muscle occurs. Mean fiber length for the region of the closer sampled is proportional to the maximum thickness of the manus. The square of mean fiber diameter for this region is proportional to a measure of the area over which fibers attach. The latter result suggests that the number of fibers in a closer muscle is nearly constant during growth.

The spectrum of sarcomere lengths in the sampled region is bimodal in most muscles, though fibers with short sarcomeres were absent from some samples. The length and fraction of long sarcomeres increase more slowly, but over a longer period, in chelipeds than in walking legs. In chelipeds fibers elongated by addition of sarcomeres as well as by elongation of sarcomeres.

The closer muscles of homoeotic legs resemble the closer muscles of normal donor-type legs of the same size. A walking leg regenerated from the coxopodite of a cheliped grows larger in external dimensions and in closer muscle mass, fiber length, and fiber diameter than a normal walking leg. A cheliped regenerated from the coxopodite of a walking leg is smaller than a normal cheliped in these parameters. Thus the host site modulates the growth rate of the homoeotic leg, externally and internally, toward the growth rate of the leg normally present there.

INTRODUCTION

Muscle fibers develop through the interaction of intrinsic properties of myoblasts with extrinsic agents, including motoneurons and attachment surfaces (Finlayson, 1975). In a homoeotic appendage muscle fibers attach to surfaces in the abnormal appendage but are subject to the remote controls (motoneurons, blood supply)

Received June 15, 1979; accepted September 25, 1980.

Abbreviations: C, cheliped; W, walking leg; K, mean ratio of fiber thickness to fiber diameter; s_{long}, mean length of long sarcomeres.

[1] This paper is dedicated to the memory of Fred Lang, whose untimely death impoverishes the science and the lives of those who were his friends and colleagues.

[2] Present address: Department of Biology, University of Oregon, Eugene, Oregon 97403.

normal for a different appendage. Therefore the traits of muscles in a homoeotic appendage should suggest what contributions attachment surfaces and remote controls make to muscle properties.

In arthropods muscle fibers attach to the epidermis, which also controls external form. Thus homoeosis of external form must modify the influence of attachment surfaces on muscle fibers. Motoneurons may influence muscle properties in Crustacea (Atwood, 1973), as in vertebrates (Close, 1972). Therefore homoeotic regenerated legs of crayfish (Mittenthal, 1980) allow analysis of extrinsic modulation of muscle development.

Wiersma (1955) found that the closer muscles of the cheliped and first walking leg in the lobster *Homarus vulgaris* differed in the number of impulses required to elicit noticeable closing of the dactylopodite. The present work shows that the homologous muscles of crayfish differ in the distributions of fiber length, fiber diameter, and sarcomere length. For a given type and size of leg these distributions are similar, whether the leg develops at the cheliped site or the walking leg site. Thus in the homoeotic legs the determinants of leg type carried with the graft dictate the morphology of muscle fibers that regenerate. However, the host site alters the size of the leg and of its muscle fibers, keeping the proportions of muscles to leg nearly normal.

Materials and Methods

Transplantation

Crayfish were cultured and operated as described by Mittenthal (1980). For legs from which closer muscles were assayed, a basipodite bearing a small limb bud (0–2 mm) was grafted. Therefore the donor site may have influenced the regenerated muscles. However, the limb bud typically became necrotic after the interchange operation (Mittenthal, 1980). Extensive de- and re-differentiation of structures in the limb bud, including muscles, probably occurred after operation.

Selection of legs for analysis

Two criteria were used to select regenerated legs for analysis of closer muscles. The leg resembled a normal cheliped or walking leg in its qualitative proportions and discrete surface markers; and the leg was used in a manner typical for the host site (spontaneous behavior, tactile closing reflex). In four animals the ventral nerve cord and its roots were exposed when legs were removed, to investigate innervation of the regenerated legs. In all these cases visual inspection showed nerves joining the ganglion of the host segment, and not of the donor segment, to the regenerate. Transection of the connectives on either side of the host segment ganglion did not abolish the closing reflex, but cutting the nerves from this ganglion to the leg abolished the motility of the leg, in the two animals thus tested.

Histology

Closer muscles were fixed according to a procedure modified from that of Lang *et al.* (1977). After autospasy the leg was immersed at 0°C in crayfish saline (van Harreveld, 1936) with 10 mM Tris replacing bicarbonate, pH 7.0 at 25°C. Ca^{2+} was reduced to 0.25 of normal (to 2.4 mM); this greatly weakens or abolishes contraction of crayfish muscle in response to nerve stimulation (Mittenthal, unpublished observation).

The carpopodite-propodite joint was transected. The propodite and dactylopodite were firmly wired to a stainless steel screen with the dactylopodite in the maximally open position. The propodite was perfused through a syringe needle inserted near the index-manus junction; the perfusate filled the up-tipped propodite and flowed from its proximal end. After perfusion for 10 min with 1/4 Ca saline at room temperature, the perfusate was changed continuously over 10–15 min to 10% formaldehyde in 1/4 Ca saline, pH 7.2. A smoothly increasing concentration of formaldehyde was mixed in an enlarged version of a linear gradient maker (Blattner and Abelson, 1966). The propodite was immersed in, and perfused with, the 10% formaldehyde saline for at least 2 hr. The closer muscle was then exposed by scraping away exoskeleton on the anterior face of the walking leg or the homologous face of the cheliped. Only fibers from this side of the pinnate closer muscle were subsequently analyzed. The muscle was resubmerged in 10% formaldehyde saline at 5° C for 48 hr, then removed from the propodite in 1/4 Ca saline, superficially dried by light blotting, weighed in 1/4 Ca saline, and stored in 70% ethanol.

Fibers were sampled from the distal dorsal region of the closer muscle (distal 1/2, dorsal 1/3). This region and its subdivisions are defined on the apodeme, not at the superficial attachment surface of the muscle. With electrolytically sharpened tungsten needles an equal number of fibers (from 4 in some muscles to 10 in others) were teased from each of the six subdivisions shown in Table III. The length and diameter of each fiber were measured with an ocular micrometer in a binocular microscope. The fibers are roughly rectangular in cross section; fiber diameter was estimated as the average of the maximum and minimum widths of a fiber lying on its broader side.

Sarcomere lengths were measured from phase contrast photomicrographs of fibers (walking leg) or of bundles of myofibrils freed by teasing (cheliped). Three regions of each fiber were photographed; in each region a sarcomere length was estimated from the length of 5–15 (typically 10) sarcomeres in series in a myofibril. If the three estimates of sarcomere length thus obtained did not agree within 25% of the minimum estimate, data for that fiber were not used. Franzini-Armstrong (1970) found a maximum variation of 25% in sarcomere lengths within single fibers of a limb muscle in the crab *Portunus*. Only one fiber of more than 750 fibers measured failed to meet this criterion. This uniformity of sarcomere length suggests that localized contraction in parts of fibers during fixation was rare.

RESULTS

Cheliped-like (C-like) or walking-leg-like (W-like) legs which regenerated at the site of a normal cheliped (C-site) will be called C-like C and W-like C, respectively. Similarly, C-like W and W-like W signify regenerated legs at the site normal for a walking leg. Normal C and W are unoperated.

Closer muscles

Fibers of the closer muscle attached to a similar zone of epidermis in all legs of a given type (C-like or W-like). A closer muscle grows approximately in proportion to its propodite (Fig. 1A); the closer in C-like legs occupies about 40%, and in W-like legs about 20%, of the volume of a rectangular parallelepiped

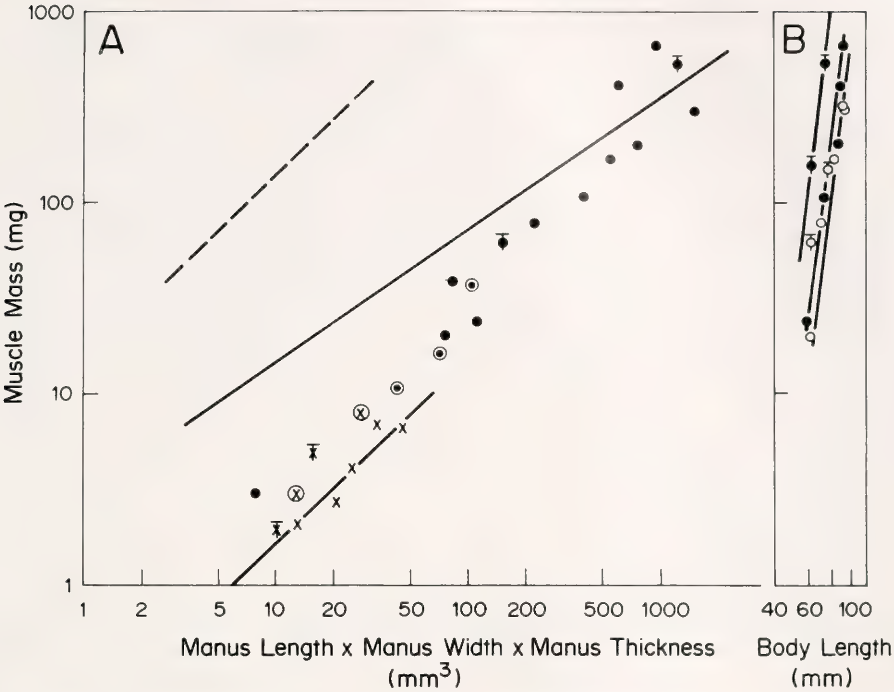


FIGURE 1: A. Mass of closer muscle *vs.* volume of a rectangular parallelepiped enclosing the manus. Filled circle: normal C and C-like C; ring around filled circle: C-like W; x: normal W and W-like W; ring around x: W-like C. T over a symbol designates normal limb of an operated animal. No difference between males and females was evident. Lines were fitted to data for unoperated animals with a nonlinear regression program (Dixon, 1975) for $y = ax^b$. For 11 chelipeds, $a = 2.9 \pm 6.1$, $b = 0.69 \pm 0.31$; for 5 walking legs, $a = 0.21 \pm 0.21$, $b = 0.91 \pm 0.27$. If muscle mass is proportional to the volume of the parallelepiped, $b = 1$. The dashed line represents $y = ax^b$ with $b = 1$. B. Mass of closer muscle *vs.* body length for chelipeds. Filled circle: male; open circle: female. Three parallel lines have been drawn by eye through points for males, females, and normal chelipeds of operated males.

that contains the manus. The closer of a normal C is 40–100 times more massive than the closer of the normal W in the same animal.

The host site alters the size of a homoeotic leg: W-like C are slightly larger than normal W, and C-like W are smaller than normal C (Mittenthal, 1980). The relations between closer mass and manus volume for normal legs roughly predict the closer mass for homoeotic legs. However, the ectopic host site affects closer mass slightly more than manus volume; two W-like C have larger closer mass, and three C-like W have smaller mass, than expected for normal propodites of the same size.

Because chelipeds of different sizes are geometrically similar, cheliped closer mass should be proportional to the cube of propodite length. The propodite of a normal cheliped is about 1.25 times longer in unoperated males, and as much as 1.7 times longer in operated males, than in unoperated females of the same body length (Mittenthal, 1980). Muscle mass might therefore be 1.95 times larger in unoperated males, and as much as 4.9 times larger in operated males, than in unoperated females. A plot of muscle mass vs. body length (Fig. 1B) showed measured factors of increase about 1.75 and 7.3. Thus, after the grafting operation

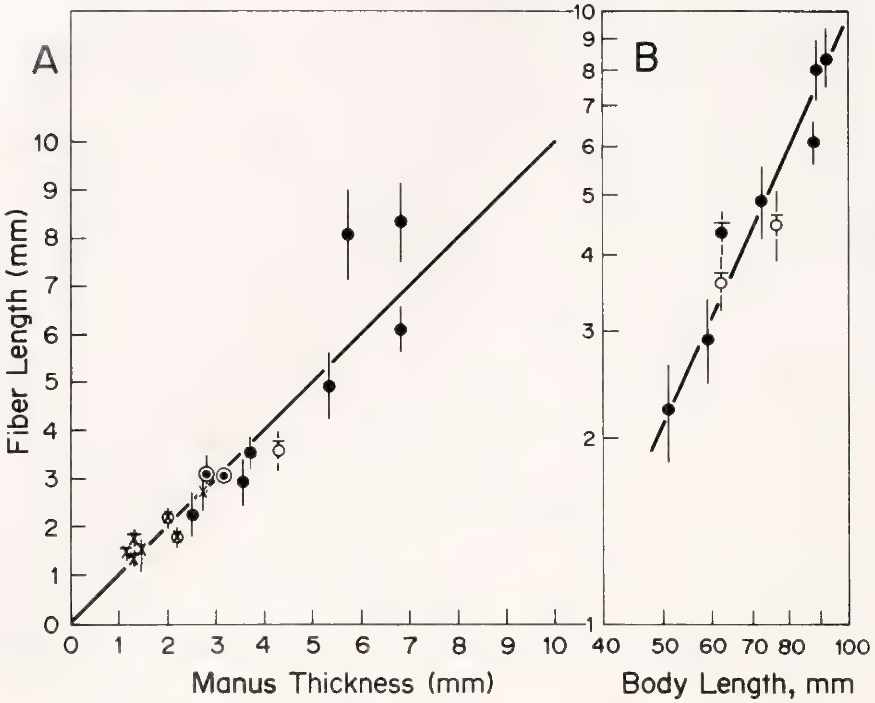


FIGURE 2: Mean±s.d. of length of fibers from distal dorsal region. A. Fiber length *vs.* manus thickness. Symbols as in Figure 1A. The line has slope 1 and passes through the origin. B. Fiber length *vs.* body length for chelipeds. Symbols as is Figure 1B. Straight line is drawn by eye through data for unoperated animals.

the unoperated cheliped grew more rapidly in closer mass than in external size, relative to the cheliped of an unoperated animal.

Dimensions of fibers

The length and diameter of a muscle fiber allow estimation of its volume and cross-sectional area. These parameters of fiber size are correlated with the size of the leg. In the closer, a pinnate muscle, the fibers extend from a central apodeme to opposite surfaces of the leg. Fiber length should increase with the distance between these surfaces—that is, with manus thickness. The mean length of fibers from the distal dorsal region is roughly equal to the manus thickness, in C-like and W-like legs at either site (Fig. 2A).

If the cross-sectional area of each fiber increases in proportion to the area of the apodeme, and if the number of fibers remains constant during growth, then fiber cross-sectional area should be proportional to the product of manus width and manus thickness. If a cross-section of each fiber retains constant proportions as the fiber grows, then its area is proportional to the square of fiber diameter. As these assumptions predict, fiber diameter is proportional to (manus length × manus width)$^{\frac{1}{2}}$ in C-like and W-like legs at both sites (Fig. 3A).

These results allow estimation of the ratio of the number of closer muscle fibers in a cheliped and in a walking leg. Approximately,

$$\frac{\text{muscle mass}}{\text{manus envelope volume}} = \frac{\begin{pmatrix}\text{number}\\ \text{of fibers}\end{pmatrix} \cdot \begin{pmatrix}\text{mean fiber}\\ \text{length}\end{pmatrix} \cdot \begin{pmatrix}\text{mean fiber}\\ \text{diameter}\end{pmatrix}^2 \cdot K}{\text{manus (length} \cdot \text{width)} \cdot \text{manus thickness}}.$$

K is the mean ratio of fiber thickness to fiber diameter. In the distal dorsal region of the closer, two ratios are the same in C and W: the ratio of mean fiber length to manus thickness, and the ratio of the square of mean fiber diameter to the product of manus length and width. If these ratios and K were known for an entire muscle, the number of fibers in the muscle could be estimated. To estimate the ratio of the fiber numbers in C and W, we assume that the above two ratios and K are the same in C as in W if fibers from the entire closer muscle are sampled. Then the ratio of the number of closer muscle fibers in C and in W is the same as the ratio, for C–W, of muscle mass to manus envelope volume. Since muscle mass divided by manus envelope volume equals approximately 0.4 in C and 0.2 in W, a cheliped closer has about twice as many muscle fibers as a walking-leg closer. Bittner and Traut (1978) showed by counting fibers that a cheliped opener muscle also has about twice as many muscle fibers as a walking-leg opener (265/135 = 1.96). The opener and closer might both have twice as many fibers in a C as in a W if the serially homologous apodemes were twice as large in a C as in a W at the time muscle fibers formed, and if newly formed fibers had the same diameter spectra in homologous muscles.

The relations between fiber size and manus size are slightly abnormal in operated animals. In C-like W the mean fiber diameter is disproportionately small (Fig. 3A). Although the mean fiber lengths in C-like W are normal relative to

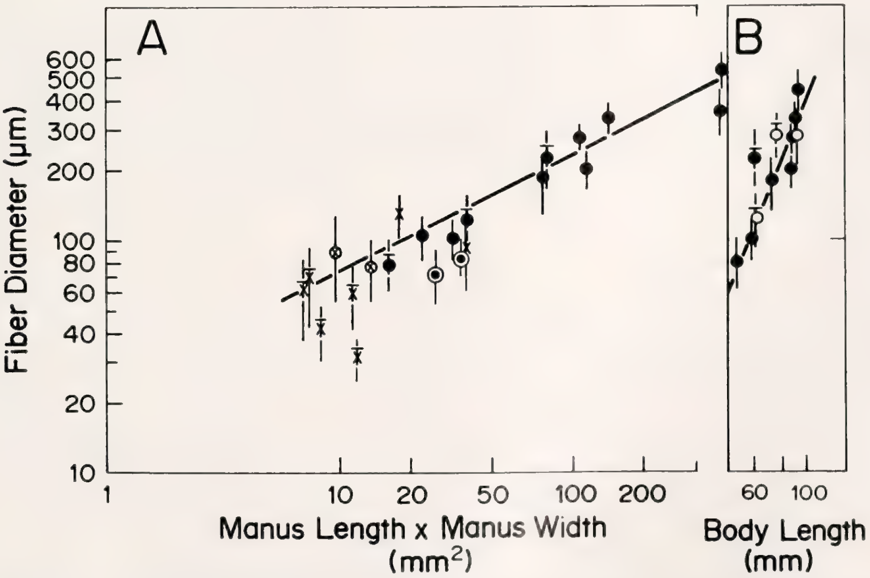


FIGURE 3: A. Mean±s.d. of diameter of fibers from distal dorsal region *vs.* manus length X manus width. Symbols as in Figure 1A. The line, fitted by nonlinear regression (*cf.* Fig. 1) has a = 22.3±1.6, b = 0.50±0.01, for 48 fibers. B. Fiber diameter *vs.* body length for chelipeds. Symbols as in Figure 1B.

TABLE I

Fiber diameter (μm) in several legs of an animal; mean ± s.d. (number of fibers). Values separated by asterisks correspond to histograms which differ significantly according to the Kolmogorov-Smirnoff test (Blalock, 1960) with P = *0.01; width of a histogram column (bin) = 20 μm.*

Leg type	62 mm Female	62 mm Male	76 mm Female	70 mm Female
Normal C	123 ± 30	227 ± 60	281 ± 58	
	*			
C-like C	105 ± 23			
	*	*	*	
C-like W	71 ± 19			
W-like C		90 ± 35	77 ± 23	
W-like W				33 ± 12
Normal W	60 ± 22	67 ± 24	58 ± 17	41 ± 11

manus thickness, the manus thickness is abnormally low (Mittenthal, 1980). In W-like C the closer mass is disproportionately large; scatter in the data may conceal a corresponding enlargement of fibers. The mean fiber length and diameter, as well as the closer mass, are disproportionately large in the unoperated cheliped of an operated animal (Figs. 1B–3B).

The ectopic host site modulates the growth of a homoeotic leg. In a given crayfish C-like and W-like regenerated legs tend to have mean fiber diameters and lengths intermediate between those of the contralateral unoperated C and W (Tables I and II). However, a homoeotic leg deviates further from the unoperated donor-type leg than does a donor-type leg regeneraed at the donor site.

In summary, the foreign host site for a homoeotic leg biases the diameter and length of closer fibers away from donor-like values and toward host-like values. Surgery and regeneration alone do not suffice to produce this bias. The host site biases muscle morphology and external dimensions together, in such a way that muscle parameters remain related to measures of external size in homoeotic legs as in unoperated legs. The changes in muscle parameters slightly exceed those in external size.

TABLE II

*Fiber length (mm) in several legs of an animal; mean ± s.d. (number of fibers); * : as in Table I; bin width = 50 μm.*

Leg type	62 mm Female	62 mm Male	76 mm Female	70 mm Female
Normal C	3.62 ± 0.36	4.36 ± 0.34	4.47 ± 0.55	
C-like C	3.53 ± 0.33			
		*	*	
C-like W	3.08 ± 0.34			
W-like C		1.75 ± 0.19	2.18 ± 0.17	
	*	*	*	
W-like W				1.41 ± 0.33
Normal W	1.44 ± 0.11	1.29 ± 0.32	1.99 ± 0.18	1.37 ± 0.22

Sarcomere length

The distribution of sarcomere lengths in the distal dorsal region of the closer muscle is usually bimodal. In animals of 50–110 mm body length most of the shorter sarcomeres are 2–4 μm long (short), but some are 4–6 μm (intermediate; Fig. 4). As an animal grows the fraction of sampled cheliped fibers having shorter sarcomeres tends to decrease toward zero (Fig. 5). In walking legs from the same animals the fraction of short sarcomeres tended to be intermediate between the fractions for chelipeds of small and large animals. It is unclear whether short sarcomeres were absent from the distal dorsal region or were missed in sampling from some legs.

Most fibers with shorter sarcomeres were in proximal subdivisions of the distal dorsal region (Table III). The non-uniformity of distribution appears especially striking in the cheliped. However, the apparent difference in distribution between chelipeds and walking legs might result from the different choice of subdivisions in the two types of legs.

As Figure 4 shows, the mean length of long sarcomeres, $\bar{s}_{\text{long}}$, tends to increase with manus length for normal chelipeds. The increase in sarcomere length is insufficient to account for the elongation of muscle fibers; mean fiber length increases more than threefold (Fig. 2A) while $\bar{s}_{\text{long}}$ only increases by about 50%. Hence fibers of the cheliped closer elongate both by elongation of sarcomeres and by adding new sarcomeres.

Recently Bittner and Traut (1978) have suggested that the variation in number of sarcomeres per fiber with fiber length cannot be deduced satisfactorily for large

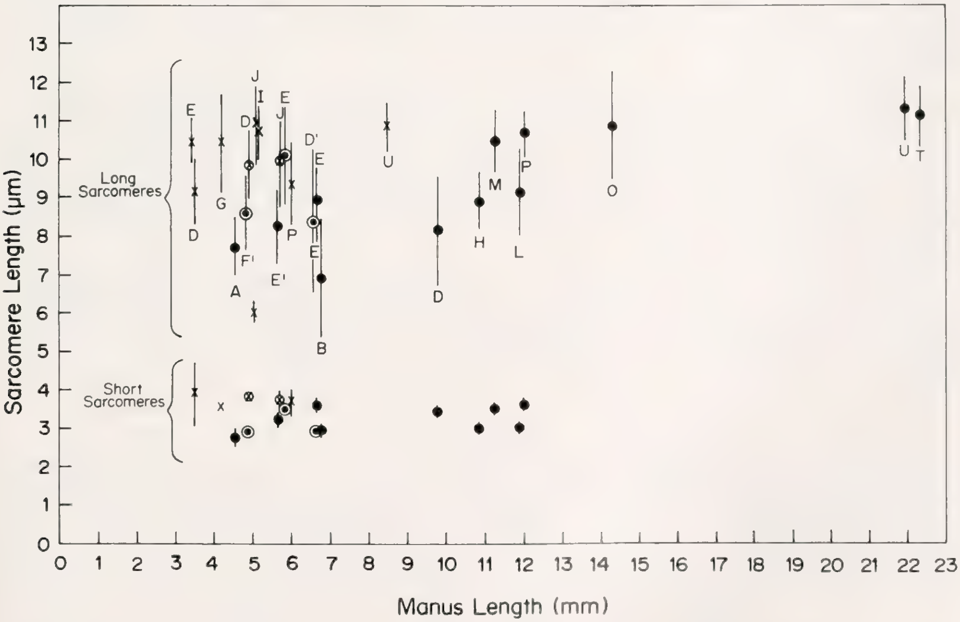


FIGURE 4: Sarcomere length of fibers in the distal dorsal part of the closer muscle, *vs.* manus length. Mean±s.d. calculated separately for long and for short sarcomeres. Letters A–U designate particular animals; body length increases with position of letter in alphabet. Filled circle: normal C and C-like C; ring around filled circle: C-like W; x: normal W and W-like W; ring around x: W-like C.

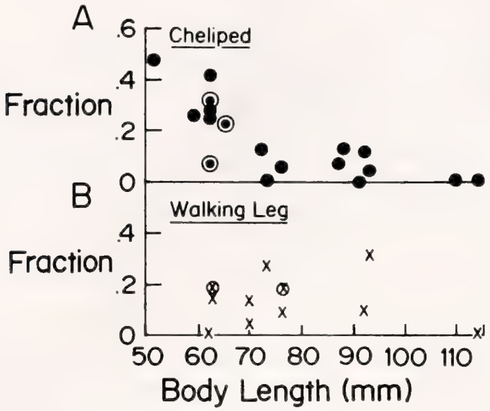


FIGURE 5: Fraction of fibers with short sarcomeres *vs.* body length. Symbols as in Figure 4.

regions of a muscle from plots of fiber length and sarcomere length vs. external dimensions (Figs. 2, 4). Dispersion in sarcomere length may obscure constancy of the number of sarcomeres per fiber as the fibers grow. We have therefore estimated the number of sarcomeres per fiber as the mean of the ratio of fiber length to sarcomere length, for all long-sarcomere fibers sampled from each of the six subdivisions of the distal dorsal region, in chelipeds of six normal animals. These estimates, in Table IV, show that the number of sarcomeres per fiber tends to increase with size of the crayfish in all six subdivisions and in the distal dorsal region as a whole. Thus cheliped closer fibers lengthen by addition as well as by elongation of sarcomeres.

The relative contributions to elongation of walking-leg fibers by elongation and by addition of sarcomeres are unclear because of scatter in the data. The $\bar{s}_{long}$ values for walking legs cluster near the maximum $\bar{s}_{long}$ in large chelipeds. The ratio of $\bar{s}_{long}$ values for normal C and W of one animal shifts from less than 1 for small crayfish (<65 mm; animals D, E) to greater than 1 in large crayfish (>90 mm long; animals J, P, U).

In homoeotic legs (two W-like C, three C-like W), $\bar{s}_{long}$ has roughly the value expected for a normal propodite of donor type with the observed manus length. Unlike mean fiber length and diameter, $\bar{s}_{long}$ for a homoeotic leg was not clearly displaced toward the value for the contralateral host-type leg.

TABLE III

Distribution in normal chelipeds and walking legs of sampled fibers having short sarcomeres, among the subdivisions of the distal dorsal region. Data for unoperated and operated animals are pooled, as no difference was noted. A. In normal chelipeds, fraction of 60 fibers from nine animals in the subdivisions. B. In normal walking legs, fraction of 15 fibers from four animals.

A.

		Dorsal		
Proximal	0.20	0.067	0	Distal
	0.60	0.117	0.017	
		Ventral		

B.

0.333	0.333	0	0
	0.2	0.133	

TABLE IV

Number of sarcomeres per fiber. Mean ± s.d. (number of fibers) of fiber length/mean sarcomere length for the six subdivisions of the distal dorsal region, and for the entire region, in chelipeds of six unoperated male animals.

Body length (mm)	51	59	72	87	88	91
Manus length (mm)	4.54	6.76	10.8	11.9	11.2	14.3
Subdivision 1	204 ± 19 (3)	376 ± 64 (5)	529 ± 34 (5)	544 ± 32 (5)	709 ± 69 (5)	673 ± 19 (6)
2	223 ± 24 (5)	339 ± 106 (5)	441 ± 6 (5)	560 ± 25 (5)	734 ± 80 (5)	710 ± 34 (6)
3	311 ± 63 (3)	480 ± 114 (5)	587 ± 44 (5)	730 ± 112 (5)	737 ± 42 (5)	713 ± 80 (6)
4	280 ± 26 (5)	592 ± 48 (3)	564 ± 69 (5)	642 ± 39 (5)	817 ± 19 (5)	773 ± 70 (6)
5	371 (1)	387 ± 119 (5)	517 ± 63 (5)	815 ± 47 (5)	696 ± 126 (5)	819 ± 132 (6)
6	— (0)	— (0)	604 (1)	790 ± 73 (3)	899 (1)	1006 ± 97 (6)
Distal dorsal region	261 ± 57 (17)	421 ± 122 (23)	530 ± 68 (26)	672 ± 121 (28)	745 ± 86 (26)	783 ± 135 (36)

DISCUSSION

Transplantation has been used extensively to study the development of motor systems in vertebrates (*e.g.*, Detwiler, 1964; Hollyday *et al.*, 1977). However, in arthropods similar methods have been restricted to insects (Young, 1972; Westin and Camhi, 1975a, b). Recently a method for transplanting regenerating crustacean limb buds has been applied to motor systems in fiddler crabs (Trinkaus-Randall and Mittenthal, 1978) and in crayfish, as reported here. The effects of an ectopic host site on muscles in homoeotic legs will be discussed after a summary of the post-embryonic growth of crustacean muscle in normal legs.

A muscle may grow by an increase in the number or in the dimensions of its fibers. In vertebrates, fibers usually grow in length and cross-sectional area but remain constant in number as the animal grows (reviewed by Goldspink, 1972). However, in hypertrophy of muscles induced by exercise the number of fibers in the muscle has been observed to increase significantly (Gonyea et al., 1977).

The number of fibers in the opener muscle of the crayfish cheliped is constant at about 300, in normal and regenerated chelipeds having a 10-fold range of propodite lengths (Bittner, 1968). Bittner found that fiber length and fiber diameter increase linearly with propodite length. The dry mass of the opener muscle increased with propodite length in the way expected if the number of fibers remains constant and if the propodite maintains constant proportions as it grows.

The measurements of propodite dimensions reported by Mittenthal (1980) confirm Bittner's assumption that chelipeds of different sizes are geometrically similar. Our findings on the closer muscles of the cheliped and first walking leg are consistent with Bittner's observations and assumptions for the cheliped opener: The wet mass of the fixed closer muscle is proportional to the volume of a box enclosing the manus, which contains the closer and opener. The mean length and diameter of closer fibers are proportional to linear dimensions of the manus. The latter proportionality means that the number of fibers remains nearly constant during growth.

However, Davison (1956) found that abdominal flexor muscle of *Procambarus alleni* grows by addition of new fibers as well as by enlargement of extant fibers.

Histological examination of the muscles showed aggregation and fusion of myoblasts. Over a 4000-fold range of body weight, the number of fibers increased about 5-fold for a 1000-fold increase in weight.

If the number of fibers in the closer muscle increased at the rate found by Davison, the data of Fig. 3A should fit a straight line of slope 0.33 rather than the observed slope of 0.5. Evidently some crustacean muscles, such as the opener in a crab (Lang, Sutterlin, and Prosser, 1970) grow only by enlargement of fibers. However, others, such as abdominal flexors of crayfish and the stretcher muscle of lobster pereiopods (Jahromi and Atwood, 1971a) also grow by increasing the number of fibers. Differences in the structure or function of muscles correlated with these two modes of growth are unknown.

As a crustacean muscle fiber elongates, the number and/or length of its sarcomeres increases (Bittner, 1968; Goudey and Lang, 1974; Govind *et al.*, 1974; Jahromi and Charlton, 1979). In some crustacean muscles fibers grow mainly or solely by elongation of sarcomeres (Bittner and Traut, 1978). However, in other muscles sarcomeres elongate and new sarcomeres are added (Govind *et al.*, 1977). Fibers of the cheliped closer muscles in *P. clarkii* grow in both ways. Mean sarcomere length increases slightly with manus length in chelipeds, but much of the increase in fiber length is contributed by addition of sarcomeres. Growth of sarcomeres and an increase of their number during elongation of fibers have also been observed in other arthropods (Aronson, 1961; Shafiq, 1963; Auber, 1965) and in vertebrates (Goldspink, 1968; Williams and Goldspink, 1971).

Muscles in several species of decapod crustaceans have bimodal distributions of sarcomere length (*Procambarus*: present work; lobster, *Homarus*: Jahromi and Atwood, 1971b; snapping shrimp, *Alpheus*: Stephens and Mellon, 1979a). In crayfish the spectrum of sarcomere lengths in the walking leg attains its asymptotic character early in development, while slow changes in the spectrum continue in the cheliped. A parallel situation occurs in small juvenile lobsters: The closer muscle of the cutter has nearly attained the adult fraction of short sarcomeres by stage 11, whereas the crusher is not yet adult-like at stage 16 (Govind and Lang, 1978). Evidently in the crayfish and lobster, the closer muscles of the larger, more specialized types of leg (crayfish cheliped, lobster crusher) have evolved in part through a prolongation of growth processes that proceed more rapidly and stop earlier in the smaller, less specialized legs. A slowing and prolongation of growth often accompanies the evolution of larger, more specialized structures (Gould, 1977).

In the present work measurements of propodite dimensions, muscle mass, and fiber morphology in normal legs provide a background for evaluating the contribution of the host site to the properties of homoeotic legs. The homoeotic legs resembled donor legs in muscle morphology as well as external form. The host site produced a slightly greater alteration of size in muscle fibers than in external form.

Similar grafting experiments in fiddler crabs also yielded regenerated legs with donor-like external form and muscles. In a large cheliped regenerated on a male specimen of *Uca pugnax* from the basipodite of a male specimen of *Uca pugilator*, sarcomere lengths of fibers from the major carpopodite extensor muscle were those expected for a donor cheliped (*ca.* 10 μm) rather than a host cheliped (13–14 μm) (Trinkaus-Randall and Mittenthal, 1978).

It is noteworthy that in some circumstances the host site can modulate the external form and muscle properties of a leg. In *Alpheus* loss of the snapper cheliped causes reversal of cheliped asymmetry: The pincer transforms to a snapper, and a pincer regenerates in place of the former snapper. Recent studies of cheliped muscles (Stephens and Mellon, 1979a, b; Mellon and Stephens, 1978, 1979) show that the closer muscles of snapper and pincer have homologous innervation but differ in spectra of fiber length, fiber diameter, and sarcomere length, and in the size and facilitatory characteristics of excitatory junctional potentials. When a pincer is transformed to a snapper, these muscle properties are transformed correspondingly. Evidently the site where a leg develops, and interactions between adjacent legs, can modulate the type of leg that develops, evoking a co-ordinated transition of external form and muscle properties.

Recent work on the development of lobster chelipeds shows that the normal co-ordination between external form and muscle properties can be disrupted. In *Homarus* the closer muscles of crusher and cutter chelipeds have different distribution of sarcomere lengths. Normally the cutter closer has about 70% short sarcomere fibers (2–4 μm sarcomere length); 30% of the fibers have sarcomeres greater than 6 μm long (Jahromi and Atwood, 1971b). Lang *et al.* (1978) found that lobsters raised in smooth-bottomed tanks retained two cutter-like chelipeds. One of these chelipeds had 90% short-sarcomere fibers; the other, a "false" cutter, had 50–60%. Govind and Lang (1979) examined lobsters having paired crusher-like chelipeds. In one of these chelipeds the closer resembled a normal crusher closer, with no short sarcomeres. However, the contralateral cheliped, though crusher-like in external form, had a closer with about 40% short sarcomere fibers. These observations show that the closer muscles of lobster chelipeds tend to be asymmetric, even when the chelipeds are externally symmetric.

The preceding discussion suggests a model for the control of leg development in decapod Crustacea. During ontogeny separate control systems may generate the external form and muscle properties characteristic of each leg. Normally the hypodermis and muscle generators are co-ordinated, in a way not understood, to generate typical legs. Errors in coupling the two generators may occur, so that the hypodermis generator typical of one leg and the muscle generator typical of another leg must co-operate to produce a leg. Apparently this happens in lobsters with symmetric chelipeds. Although the hypodermis and muscle generators play a major role in determining leg characteristics, the site on the body where a leg develops and the control systems generating adjacent legs can modulate the development of a leg. Such modulation can occur even if the coupling between hypodermis type and muscle type is normal, as in the homoeotic crayfish legs studied in the present work.

This model has testable implications. For example, lobsters might be found in which the chelipeds appear normal externally, but the muscle asymmetry is the reverse of the external asymmetry. To screen for lobsters with reversed closer muscle asymmetry one could test the speed of the dactylopodite closing reflex, looking for animals with unusually fast crusher closing and unusually slow cutter closing.

ACKNOWLEDGMENTS

We thank Dr. Arthur Winfree and Ms. Vickery Trinkaus-Randall for a critical reading of the manuscript. We are indebted to Ms. Judith Franklin for the nonlinear regressions. Dr. Fred Lang provided invaluable advice on histology, and helpful criticism in discussions. Dr. Lang and Dr. George Bittner supplied manuscripts in press. This research was supported in part by NSF grant no. BNS77-24149.

LITERATURE CITED

Aronson, J., 1961. Sarcomere size in developing muscles of a tarsonemid mite. *J. Biophys. Biochem. Cytol.*, **11**: 147–156.

Atwood, H. L., 1973. An attempt to account for the diversity of crustacean muscles. *Am. Zool.*, **13**: 357–378.

Auber, J., 1965. L'accroissement en longeur des myofibrilles et la formation de nouveaux sarcomeres on cours du developpement des muscles chez *Calliphora erythrocephala. C.R. Acad. Sci.* (Paris), **261**: 4845.

Bittner, G. D., 1968. The differentiation of crayfish muscle fibers during development. *J. Exp. Zool.*, **167**: 439–456.

Bittner, G. D., and D. L. Traut, 1978. Growth of crustacean muscles and muscle fibers. *J. Comp. Physiol.*, **124**: 277–285.

Blattner, F. R., and J. N. Abelson, 1966. A precision method for generation of solvent gradients. *Anal. Chem.*, **38**: 1279–1281.

Blalock, H. M., 1960. *Social Statistics.* McGraw-Hill Book Co., Inc., New York. xiv, 465 pp.

Close, R. I., 1972. Dynamic properties of mammalian skeletal muscles. *Physiol. Rev.*, **52**: 129–197.

Davison, J., 1956. An analysis of cell growth and metabolism in the crayfish (*Procambarus alleni*). *Biol. Bull.*, **110**: 264–273.

Detwiler, S. R., 1964. *Neuroembryology.* Hafner Publ. Co., New York, x, 218 pp.

Dixon, W. J., 1975. BMDP: Biomedical Computer Programs. University of California Press. Berkeley, Los Angeles, xi, 792 pp.

Finlayson, L. H., 1975. Development and degeneration. Pp. 75–149 in P. N. R. Usherwood, Ed., *Insect Muscle.* Academic Press, New York.

Franzini-Armstrong, C., 1970. Natural variability in the length of thin and thick filaments in single fibers from a crab, *Portunus depurator. J. Cell Sci.*, **6**: 559–592.

Goldspink, G., 1968. Sarcomere length during the post-natal growth of mammalian muscle fibers. *J. Cell Sci.*, **3**: 539–548.

Goldspink, G., 1972. Postembryonic growth and differentiation of striated muscle. Pp. 181–236 in G. H. Bourne, Ed., *The Structure and Function of Muscle.* Second edition. Vol. 1.

Gonyea, W., C. G. Ericson, and F. Bonde-Peterson, 1977. Skeletal muscle fiber splitting induced by weight-lifting exercise in cats. *Acta Physiol. Scand.*, **99**: 105–109.

Goudey, L. R., and F. Lang, 1974. Growth of crustacean muscle: Asymmetric development of the claw closer muscles in the lobster, *Homarus americanus. J. Exp. Zool.*, **189**: 421–427.

Gould, S. J., 1977. *Ontogeny and Phylogeny.* Harvard University (Belknap) Press, Cambridge, Mass. ix, 501 pp.

Govind, C. K., H. L. Atwood, and F. Lang, 1974. Sarcomere length increases in developing crustacean muscle. *J. Exp. Zool.*, **189**: 395–400.

Govind, C. K., and F. Lang, 1978. Development of the dimorphic claw closer muscles of the lobster *Homarus americanus.* III. Transformation to dimorphic muscles in juveniles. *Biol. Bull.*, **154**: 55–67.

Govind, C. K., and F. Lang, 1979. Physiological asymmetry in the bilateral crusher claws of a lobster. *J. Exp. Zool.*, **207**: 27–32.

Govind, C. K., J. She, and F. Lang, 1977. Lengthening of lobster muscle fibres by 2 age-dependent mechanisms. *Experientia,* **33**: 35–36.

Hollyday, M., V. Hamburger, and J. M. G. Farris, 1977. Localization of motor neuron pools supplying identified muscles in normal and supernumerary legs of chick embryo. *Proc. Nat. Acad. Sci. U.S.A.* **74**: 3582–3586.

Jahromi, S. S., and H. L. Atwood, 1971a. Electrical coupling and growth in lobster muscle fibers. *Can. J. Zool.*, **49**: 1029–1034.

Jahromi, S. S., and H. L. Atwood, 1971b. Structural and contractile properties of lobster leg-muscle fibers. *J. Exp. Zool.*, **176**: 475–486.

Jahromi, S. S., and M. P. Charlton, 1979. Transverse sarcomere splitting: A possible means of longitudinal growth in crab muscles. *J. Cell Biol.*, **80**: 736–742.

Lang, F., W. J. Costello, and C. K. Govind, 1977. Development of the dimorphic claw closer muscles of the lobster *Homarus americanus*: I. Regional distribution of muscle fiber types. *Biol. Bull.*, **152**: 75–83.

Lang, F., C. K. Govind, and W. J. Costello, 1978. Experimental transformation of muscle fiber properties in lobster. *Science*, **201**: 1037–1039.

Lang, F., A. Sutterlin, and C. L. Prosser, 1970. Electrical and mechanical properties of the closer muscle of the Alaskan king crab *Paralithoides camtschatica. Comp. Biochem. Physiol.*, **32**: 615–628.

Mellon, D., Jr., and P. J. Stephens, 1978. Limb morphology and function are transformed by contralateral nerve section in snapping shrimps. *Nature*, **272**: 246–248.

Mellon, D., Jr., and P. J. Stephens. 1979. The motor organization of claw-closer muscles in snapping shrimp. *J. Comp. Physiol.*, **132**: 109–115.

Mittenthal, J. E., 1980. On the form and size of crayfish legs regenerated after grafting. *Biol. Bull.* (in press).

Shafiq, S. A., 1963. Electron microscopic studies on the indirect flight muscles of *Drosophila melanogaster. J. Cell. Biol.*, **17**: 363–373.

Stephens, P. J., and DeF. Mellon, Jr., 1979a. Modification of structure and synaptic physiology in transformed shrimp muscle. *J. Comp. Physiol.*, **132**: 97–108.

Stephens, P. J., and DeF. Mellon, Jr., 1979b. Morphology and synaptic physiology of the main closer muscle in regenerating claws of *Alpheus. J. Comp. Physiol.*, **134**: 17–27.

Trinkaus-Randall, V., and J. E. Mittenthal, 1978. Intra- and interspecific transplantation of limb buds in crustacea: A new method for studying central and peripheral interactions in crustacean limbs. *J. Exp. Zool.*, **204**: 275–281.

van Harreveld, A., 1936. A physiological solution for fresh-water crustaceans. *Proc. Soc. Exp. Biol. and Med.*, **34**: 428–432.

Wiersma, C. A. G., 1955. An analysis of the functional differences between the contractions of the adductor muscles in the thoracic legs of the lobster *Homarus americanus. Arch. Neerl. Zool.*, **11**: 1–13.

Westin, J., and J. M. Camhi, 1975a. Regeneration of peripheral nerves to supernumerary legs in cockroaches. *J. Exp. Biol.*, **63**: 483–496.

Westin, J., and J. M. Camhi, 1975b. Motor innervation within supernumerary legs in cockroaches. *J. Exp. Biol.*, **63**: 497–503.

Williams, P. E., and G. Goldspink, 1971. Longitudinal growth of striated muscle fibers. *J. Cell Sci.*, **9**: 751–767.

Young, D., 1972. Specific re-innervation of limbs transplanted between segments in the cockroach, *Periplaneta americana. J. Exp. Biol.*, **57**: 305–316.

Reference: *Biol. Bull.*, **159**: 728–736. (December, 1980)

ANNUAL GONADAL VARIATION IN SEA URCHINS OF THE ORDERS ECHINOTHURIOIDA AND ECHINOIDA

TAKAO MORI,[1] TEIZO TSUCHIYA,[2] AND SHONAN AMEMIYA[3]

[1] *Zoological Institute, Faculty of Science, University of Tokyo, Bunkyo-ku, Tokyo 113;*
[2] *Department of Physiology, School of Medicine, Teikyo University, Itabashi-ku,*
Tokyo 173; and [3] *Misaki Marine Biological Station, Faculty of Science,*
University of Tokyo, Miura-shi, Kanagawa 238-02

Abstract

The echinothurioid sea urchins, *Asthenosoma ijimai* and *Araeosoma owstoni*, were collected throughout the year. Their gonads were studied histologically by light microscopy and compared with those of some species of the order Echinoida. The ovaries contained degenerating and large developing oocytes throughout the year, showing that, unlike species of the order Echinoida so far studied, the Echinothurioid sea urchins lack the so-called resting state in which the ovaries contain only oogonial clusters. In the testes of this species, differentiation of spermatocytes into more advanced stages of male germinal cells appears to be blocked until at least 5 months before spawning. Spermatids appeared 3 months before spawning and spermatozoa at least 1 month before. Factors involved in differences in annual gonadal variation between the sea urchins of the orders Echinoida and Echinothurioida are discussed.

Introduction

Many species of sea urchins exhibit an annual reproductive cycle, with oocytes and spermatocytes becoming mature germinal cells during a few months before the breeding season (Boolootian, 1966). Although reproductive cycles have been studied in detail in some species of the orders Echinoida, Arbacioida, Clypeasteroida, *etc.* (Boolootian, 1966), little information is available on the gonadal cycles in the sea urchins of Echinothurioida. Recently, Amemiya and Tsuchiya (1979) succeeded in fertilizing eggs of the Japanese echinothurioid, *Asthenosoma ijimai*, in mid-September and followed subsequent development for 2 months. They pointed out important differences between development in this species and in the order Echinoida—differences probably ascribable to taxonomic and phylogenetic differences between the two groups. This is the first report comparing the annual reproductive cycle in this sea urchin with the cycles in some species of the order Echinoida.

Materials and Methods

Reproductive cycles of two echinothurioids, *Asthenosoma ijimai* Yoshiwara and *Araeosoma owstoni* Mortensen, were compared with those of three species of the order Echinoida, *Hemicentrotus pulcherrimus* (A. Agassiz), *Anthocidaris crassispina* (A. Agassiz), and *Pseudocentrotus depressus* (A. Agassiz).

Received June 5, 1980; accepted August 20, 1980.

Adult specimens were collected at varying intervals between April, 1977, and October, 1979, along the coast around Misaki Marine Biological Station, Kanagawa, Japan. For histological observations, the middle portion of one of the five gonads in individual urchins was removed and fixed in sea water and Bouin's fluid, dehydrated in ethanol, cleared in xylene, and embedded in paraplast. Serial sections cut at 7 μm were stained with Delafield's hematoxylin and eosin.

Five stages of the gonadal cycle were distinguished according to the criteria described by Fuji (1960), *i.e.* stage I (recovery), stage II (growing), Stage III (premature), stage IV (mature), and stage V (spent). The general morphology and staining characteristics of the ovary were used to define oocyte stages (Gonor, 1973). For quantitative studies, oocytes and mature ova were counted in five ovaries randomly taken from sea urchins of each of the three species *Asthenosoma ijimai, Hemicentrotus pulcherrimus,* and *Anthocidaris crassispina.* At 70 × magnification, all normal-appearing oocytes with germinal vesicles and mature ova were counted in every 200th section of the ovaries, with oogonia and oocytes measuring less than 15 μm in their largest diameter excluded. At least 2000 oocytes, divisible into four or five size groupings, were counted per specimen, at several different times throughout the year. Frequency distributions of oocytes and different sizes were determined from their percentages in the total oocytes counted.

RESULTS

There were no marked differences of the gonadal cycles and structures among the three species *Hemicentrotus pulcherrimus, Anthocidaris crassispina,* and *Pseudocentrotus depressus.* Around Misaki Marine Biological Station, the breeding season usually continued from January through March in *H. pulcherrimus,* from June through September in *A. crassispina,* and from October through December in *P. depressus.* Each of five separate ovaries located under the peritoneum consists of branched saccules (ovarioles) with final blind acini. Young oocytes were on the ovarian wall, which was made up of connective tissue and smooth muscle fibers. The central portion of the acinous lumen was occupied by germinal cells, which matured to ova, and accessory cells, here called nutritive phagocytes.

In *H. pulcherrimus* and *A. crassispina,* oocytes were counted in late December, 1978; late March, 1979, and late June, 1979. Oocytes were arbitrarily divided into four size classes: diameter 15–30 μm (young oocytes), diameter 30–65 μm (developing oocytes), largest diameter more than 65 μm (large primary oocytes and secondary oocytes), and mature ova. The size distributions of oocytes clearly demonstrate the annual changes in ovarian activity (Figs. 1 A, B).

The ovaries in stage I were filled with accessory cells containing eosinophilic globules and large dark granules in the cytoplasm. Some germinal cells were attached to the wall in small clusters. In stage II (growing ovaries) a large number of oocytes less than 65 μm in diameter were arranged in a single uniform layer lining the acinous wall. The acinous lumen was totally occupied by a large number of globular accessory cells. In stage III (premature ovaries), numerous large oocytes projected markedly into the acinous lumen from the ovarian wall. These oocytes eventuallly left the wall of the acini. A few mature ova about 90 μm in diameter, with small female pronuclei, were in the lumen. Ovarian acini in this stage also contained developing oocytes of varying sizes, including small young oocytes on the wall. In stage IV (mature ovaries), the acinous lumen was largely packed with mature ova, although oocytes of varying sizes and a few accessory cells were on the wall. In stage V ovaries, the acinous lumen was largely

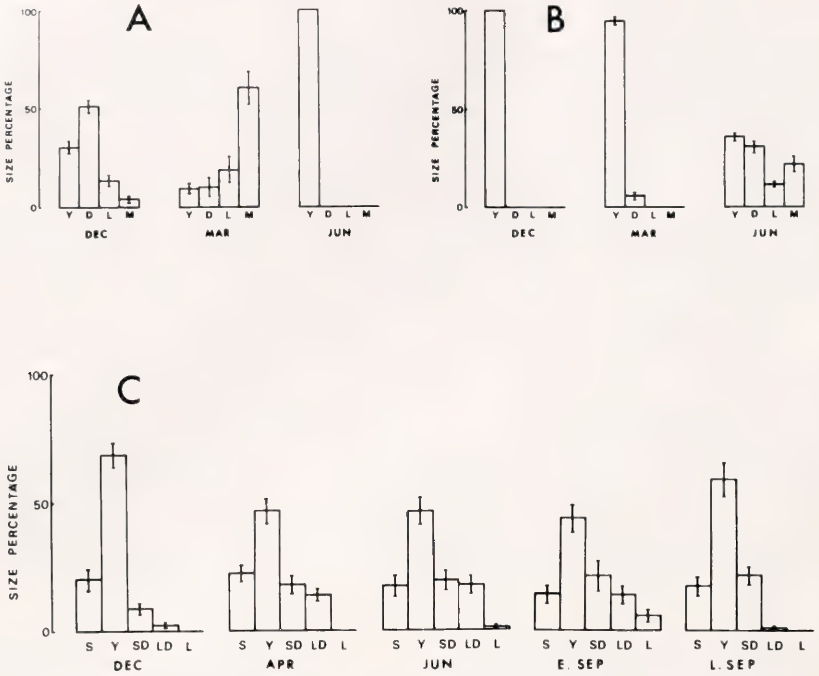


FIGURE 1. Frequency distribution of different-sized oocytes in the ovaries of *Hemicentrotus pulcherrimus* (A) and *Anthocidaris crassispina* (B), collected in December, 1978 (DEC), March, 1979 (MAR) and June, 1979 (JUN). Y = young oocytes 15–30 μm in largest diameter, D = developing oocytes 30–65 μm, L = large primary and secondary oocytes larger than 65 μm diameter, M = mature ova. (C) Frequency distribution of different-sized oocytes in the ovaries of *Asthenosoma ijimai,* collected in December, 1978 (DEC), April, 1979 (APR), June, 1979 (JUN), early September, 1979 (E. SEP) and late September, 1979 (L. SEP), S = small oocytes 15–100 μm in largest diameter, Y = young oocytes 100–400 μm diameter, SD = small developing oocytes 400–800 μm diameter, LD = large developing oocytes 800–1100 μm diameter, L = large primary oocytes over 1100 μm in diameter. Vertical lines represent standard errors.

occupied by accessory cells with vacuolated cytoplasm, but a few small oocytes measuring less than 20 μm in diameter and clusters of small germinal cells were still on the wall.

The wall of the acinous cavity of the sea urchin testis is composed of an outer visceral peritoneum, a middle fibromuscular layer composed of connective tissue and muscle fibers, and an inner layer consisting of germinal as well as non-germinal cells. In the non-breeding seasons, the acinous lumen was obliterated by proliferation of globular accessory cells. Some clusters of germinal cells were present on the acinous wall. In the premature stage spermatogenesis was very active. In almost all acini, sperm clusters had already formed in the central parts of the lumens. The acinous wall was lined by a sheet of accessory cells. In the mature stage, the testes were distended with mature spermatozoa, and some accessory cells were observable around sperm aggregations.

The echinothurioid sea urchin, *A. ijimai,* has five separate ovaries, each branching into saccules (ovarioles) ending in blind acini. However, the ovaries differ in histological structure from those of echinoid species. The echinothurioid ovaries studied were under the peritoneal epithelium, and had a wall composed of collagenous connective tissue and smooth muscle fibers. From the inner wall, thin connective tissue partitions extended interiorly, dividing the acini into several compartments containing germinal and non-germinal cells (Fig. 2A). The partitions ramify and grow toward the central part of the acinous lumen. Oocytes of varying sizes attaching to the ramified partitions are distributed without apparent order in the ovarian acini. The ovary is readily distinguishable from the testis

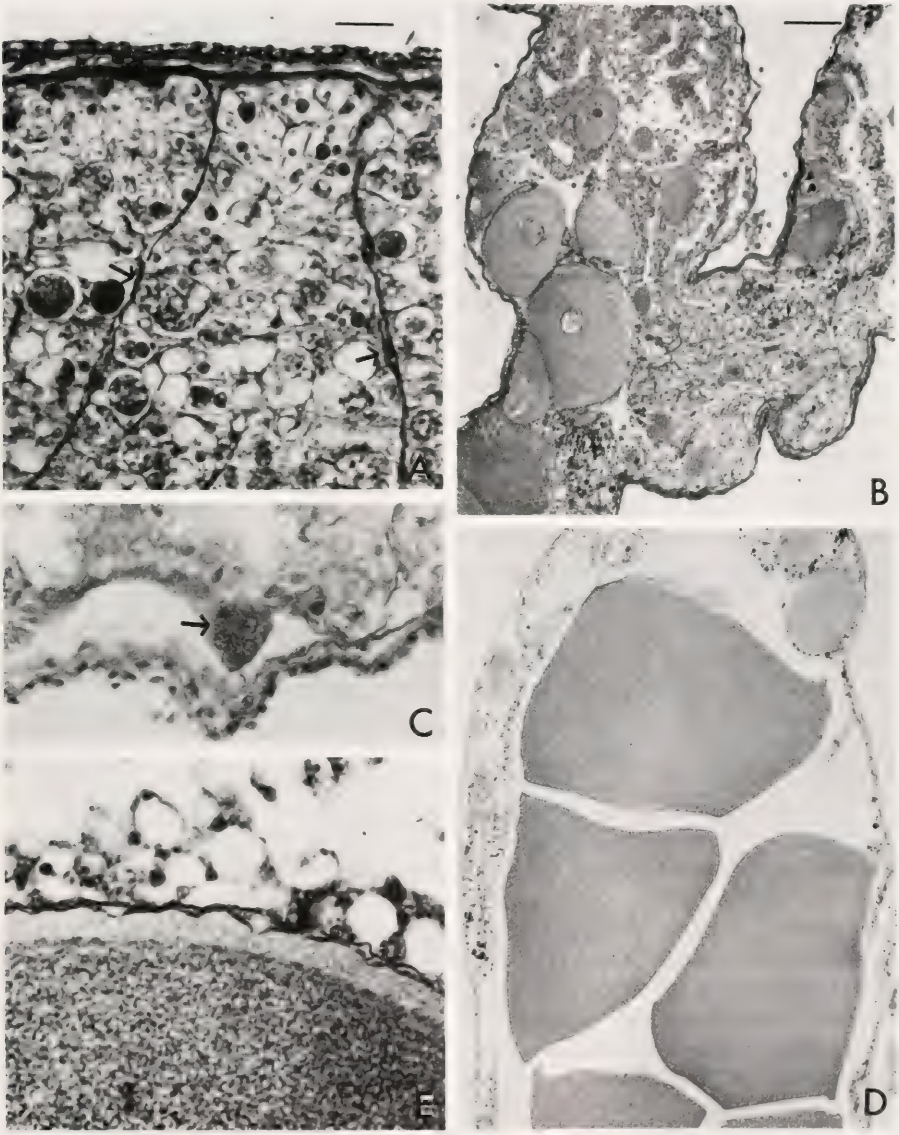


FIGURE 2. (A) Sections of ovarian lobes of *Asthenosoma ijimai*. Peripheral part of the ovarian acinus. Arrows indicate connective tissue partitions. (B) Part of ovarian acinus from a specimen collected on December 21, 1978. (C) Cluster of germinal cells (arrow) attached to ovarian wall. (D) Part of ovarian acinus from a specimen collected on September 14, 1979. (E) Cortical portion of an oocyte, undergoing dissolution. A, C, and E × 400. Scale line in A represents 20 μm. B and D × 40. Scale line in B represents 200 μm.

throughout the year (*cf.* Fig. 3), since the oocytes in the peripheral region of the ovarioles can be seen with the naked eye.

Oocytes counted in late December, 1978; early April, 1979; late June, 1979; early September, 1979; and late September, 1979, were divided into five size groupings by largest diameter: those from 15–100 μm (small oocytes), 100–400 μm (young oocytes), 400–800 μm (small developing oocytes: oocytes over 400 μm had eosinophilic granular cytoplasm, suggesting vitellogenesis), 800–1100 μm (large developing oocytes), and over 1100 μm (large primary oocytes whose dense, yolk-filled cytoplasm stained pink). Primary oocytes were not distinguishable from secondary oocytes, as reliable criteria for the species are not known. Unlike species of the order Echinoida, in *A. ijimai* the size distribution diagrams of oocytes did not show any clear annual changes (Fig. 1C), demonstrating that many small primary oocytes continue to grow throughout the year, and that oocytes of almost all sizes occur at any itme.

In December, ovaries contained oocytes of varying sizes. The largest ones, about 100 μm in largest diameter, were surrounded by vacuolated accessory cells and stained homogeneously with eosin. The large oocytes had germinal vesicles about 200 μm in diameter. The acini contained many irregular- or polygonal-shaped degenerating oocytes with large vacuoles in the cytoplasm. Oocytes over 400 μm in largest diameter were eosinophilic, while smaller ones were basophilic. Germinal vesicles of all oocytes contained a single, large, spherical, and strongly basophilic nucleolus (Fig. 2B). Clusters of small germinal cells, composed of closely packed cells of varying sizes with indistinct boundaries, were distributed over the inner connective tissue partitions (Fig. 2C). The cells sometimes appeared as aggregates of small, strongly staining nuclei, occasionally in the process of mitosis or meiosis. It was not known when the primary oocytes were produced from oogonia during the annual reproductive cycle. Globulated accessory cells were sparsely scattered in the ovarioles.

In April, the largest oocytes reached about 1100 μm in largest diameter. Many irregular-shaped degenerating oocytes were still present. In June, the ovarioles contained more oocytes and fewer accessory cells than in April. The largest oocytes measured about 1300 μm in diameter. However, ovarioles still contained degenerating oocytes. From late August through early September, ovarian sructure was almost the same as in June. Some oocytes were degenerating. Accessory cells decreased in number and almost all of them became vacuolated. Clusters of small germinal cells were arranged over the connective tissue septa.

In mid-September, the ovaries contained many large oocytes 1100–1300 μm in largest diameter. Crowded together within the narrow ovarioles, these oocytes became irregularly polygonal in shape (Fig. 2D). Careful examination of serial sections revealed that the oocytes had no nuclei. A few accesseory cells were visible on the acinous wall. Oocytes in the ovaries, which seemed to have already spawned, were invariably less than 800 μm in diameter. A decrease in number of oocytes was followed by a rapid increase in number of empty-appearing accessory cells. In late September, after the spawning season, almost all remaining oocytes were degenerating but had increased in size up to 800 μm. The degenerating oocytes had disintegrating cytoplasm and were irregular in shape. In other seasons, the egg cortex of degenerating oocytes remained relatively unchanged for a while. By constrast, in post-spawning ovaries, degeneration of oocytes began with the dissolution of the cortical layer, and the disintegration of the inner cytoplasm followed (Fig. 2E). Empty-appearing accessory cells in post-spawning ovaries increased in

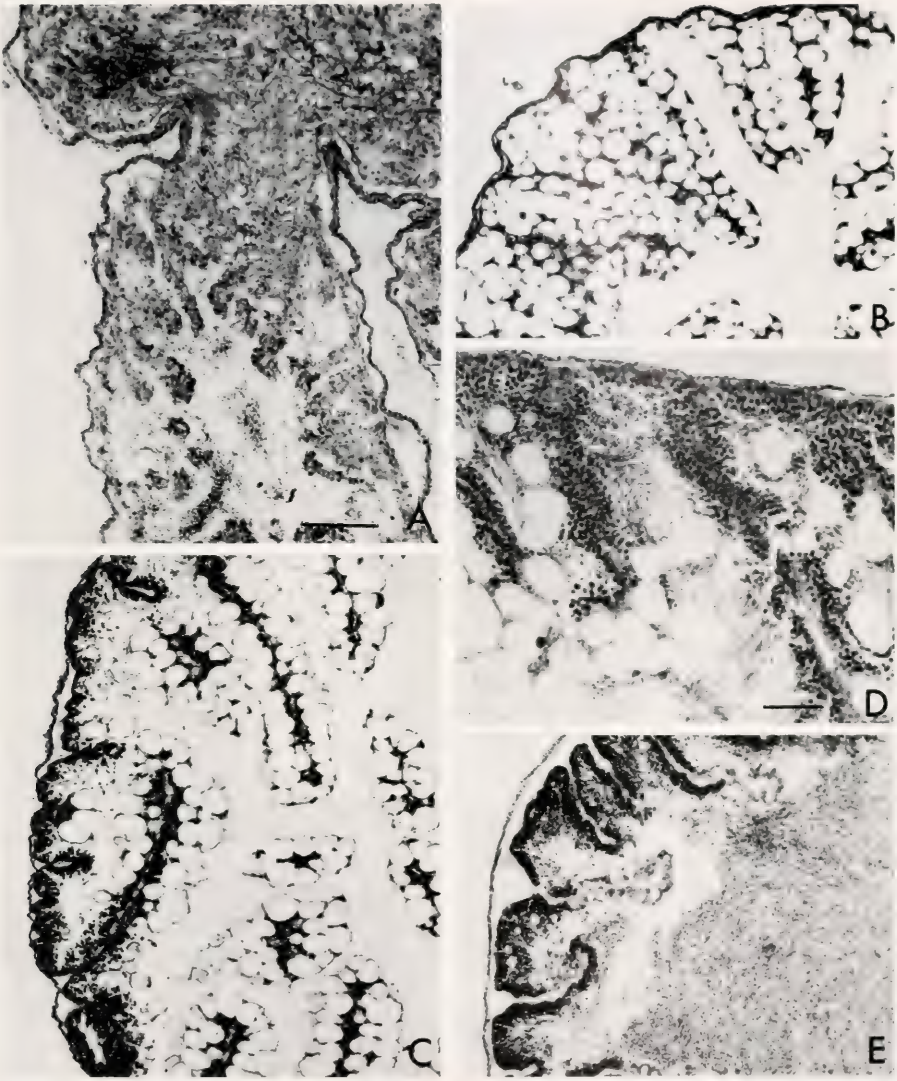


FIGURE 3. Sections of testicular lobes of *Asthenosoma ijimai*. Parts of testicular acini from specimens collected December 21, 1978 (A), April 7, 1979 (B), June 25, 1979 (C), August 14, 1979 (D) and September 12, 1979 (E). All figures except D × 100; D × 400. Scale line in A represents 100 μm and applies to A, B, C, and E; scale in D represents 20 μm.

number continuously and exhibited many dark granular inclusions. By October, oocytes measuring 400–600 μm in diameter had increased in number. The largest oocytes attained 800 μm, with germinal vesicles of 200-μm diameter. A few degenerating oocytes were also found. Accessory cells, still empty looking, increased in number.

The testicular acini of *A. ijimai* are composed of an outer visceral peritoneum, a middle fibromuscular layer, and an inner layer consisting of germinal and nongerminal cells. The inner surface of the inner layer is covered with a thin connective tissue membrane, as in the ovarioles.

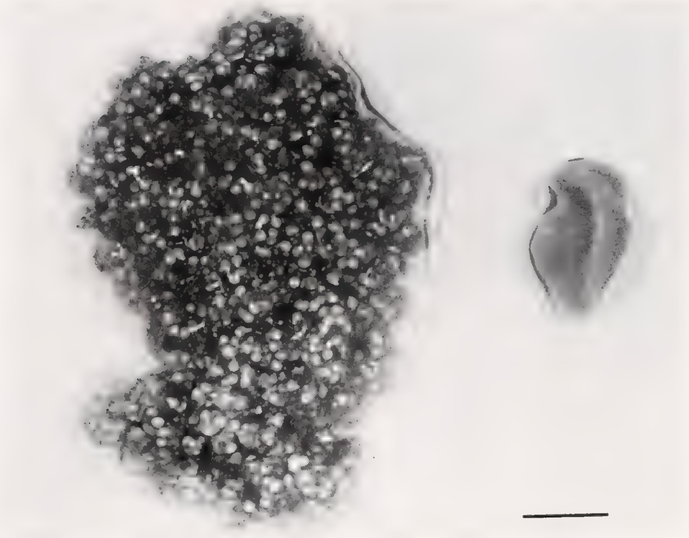


FIGURE 4. Ovaries of *Araeosoma owstoni* (left) and of *Hemicentrotus pulcherrimus* (right). 1.1 ×. Scale line indicates 1 cm.

In late December, the testes were filled with globulated accessory cells and some clusters of germinal cells located over the inner connective tissue partitions.

In some individuals, the acinous lumen contained small masses of relict sperm (Fig. 3A). In early April, the testicular acini had numerous folds on the wall extending into the acinous lumen. Vacuolated empty-looking accessory cells were arranged on this wall (Fig. 3B). Clusters of germinal cells less than 10 μm in diameter were on the inner connective tissue partitions. In late June, the lumen of the testicular acini widened (Fig. 3C). Spermatogenesis, taking place on the connective tissue partitions, resulted in production of a few spermatids in the acini, but spermatozoa were not yet present. The primary and secondary spermatocytes were not distinguishable. In August, spermatogenesis was going on even in the central part of the acini (Fig. 3D). In early September, the acinous lumen was totally occupied by mature spermatozoa and the testicular acini were greatly enlarged (Fig. 3E). In late September, the testes were approximately the same in structure as in early September, except for a decrease in numbers of spermatozoa.

Ovaries of *Araeosoma owstoni* are similar in structure to ovaries of *A. ijimai* (Fig. 4). In mid-March, the largest *A. owstoni* oocytes measured about 1000 μm in diameter and the germinal vesicles about 200 μm. Oocytes of varying sizes were distributed without apparent order in the ovarian acini. In early April, before the spawning season, the largest oocytes reached 1200 μm in diameter, with germinal vesicles measuring 340 μm. In late April, after the spawning season, the maximum diameter of the oocytes dropped to 910 μm and remained there until early October. Degenerating large oocytes were present throughout the year.

The testes of *A. owstoni* showed active spermatogenesis from March through April. In late May, spermatogenesis was still going on in the acini but empty-appearing accessory cells increased in number. From early June through early October, spermatogenesis was no longer visible, the main components of the testes being vacuolated accessory cells.

Discussion

Ovaries of the species of the order Echinoida exhibit a definite annual cycle, demonstrated by the frequency distributions of oocytes of different sizes at different times of the year (Pearse and Giese, 1966; Gonor, 1973). Mature ova and developing oocytes were totally absent during non-breeding seasons, until just before onset of the breeding season (Boolootian, 1966; Masuda and Dan, 1977; the present study). By contrast, in the echinothurioid sea urchins *A. ijimai* and *A. owstoni* no such distinct cyclic changes occurred in oocyte growth and differentiation. Small young and large developing oocytes are found in the ovaries almost throughout the year, although spawing is restricted to a short definite season. However, testes show a distinct annual activity cycle in these echinothurioid species.

Lunar periods, food, salinity, light, and temperature, among other factors, are known to influence reproductive cycles. It is particularly well established that fluctuations in quantity and quality of food are involved in changes in gonadal activity: Adequate food permits maturation of a large number of oocytes (Boolootian, 1966), while starved sea urchins (*Stronglyocentrous purpuratus*) failed to reproduce (Giese, 1959). These findings suggest that energy reserves in the gonads are important in promoting reproductive activity in the sea urchin.

The echinothurioid sea urchins dealt with in this paper live on the rocky bottom 20–30 m deep (*A. ijimai*) or on sandy bottom 80–100 m deep (*A. owstoni*), where the effects of seasonal fluctuations may be much mitigated. The intestines of the sea urchins examined contained mainly nereids, barnacles, and sponges, showing that the urchins were carnivorous. Thus they may be able to get abundant food almost year-round, in contrast to herbivorous echinoids living on seaweed that grows seasonally. This may largely account for the lack of marked rhythmic changes in ovarian morphology in the echinothurioids. However, further detailed studies are needed. Factors other than food may be largely responsible for the annual cycle of spermatogenesis in the same species.

In species of the order Echinoida, both oogenesis and spermatogenesis occur from separated germinal cells or in small groups of germinal cells within the ovarian wall of connective tissue and muscle fibers (Holland and Giese, 1965; Chatlynne, 1969; Masuda and Dan, 1977). Holland (1967) reported similar findings in the cidarid, *Stylocidaris affinis*. In species of the order Echinothurioida, extentions from the inner connective tissue wall on which young germinal cells are located grow into the central part of gonadal acini, so that young germinal cells are in the central as well as the peripheral parts of the acini and give rise to large oocytes or spermatozoa. Thus, oocytes of varying sizes are distributed throughout the lumen of the ovarian acini.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan. We wish to express our thanks to Emeritus Professor K. Takewaki of the University of Tokyo for his critical reading of the manuscript and to Dr. M. Shigei for his valuable advice. This paper is dedicated to Emeritus Professor J. Ishida of the University of Tokyo on his 70th birthday.

LITERATURE CITED

Amemiya, S., and T. Tsuchiya, 1979. Development of the echinothurid sea urchin *Asthenosoma ijimai*. *Mar. Biol.* **52**: 93–96.

Boolootian, R. A., 1966. Reproductive physiology. Pp. 561–613. in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience Publishers, New York.

Chatlynne, L. G., 1969. A histochemical study of oogenesis in the sea urchin *Strongylocentrotus purpuratus. Biol. Bull.,* **136**: 167–184.

Fuji, A., 1960. Studies on the biology of the sea urchin. I. Superficial and histological gonadal changes in gametogenic process of two sea urchins, *Strongylocentrotus nudus* and *S. intermedius. Bull. Fac. Fish. Hokkaido Univ.,* **11**: 1–14.

Giese, A. C., 1959. Reproductive cycles of some west coast invertebrates. Pp. 625–638. in R. Withrow, Ed., *Conference on Photoperiodism and Related Phenomena in Plants and Animals.* Am. Assoc. Adv. Sci., Washington, D. C.

Gonor, J. J., 1973. Reproductive cycles in oregon populations of the echinoid, *Strongylocentrotus purpuratus* (Stimpson). I. Annual gonad growth and ovarian gametogenic cycles. *J. Exp. Mar. Biol. Ecol.* **12**: 45–64.

Holland, N. D., 1967. Gametogenesis during the annual reproductive cycle in a cidaroid sea urchin (*Stylocidaris affinis*). *Biol. Bull.,* **133**: 578–590.

Holland, N. D., and A. C. Giese. 1965. An autoradiographic investigation of the gonads of the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.,* **128**: 241–258.

Masuda, R., and J. C. Dan, 1977. Studies on the annual reproductive cycle of the sea urchin and the acid phosphatase activity of relict ova. *Biol. Bull.,* **153**: 577–590.

Pearse, J. S., and A. C. Giese, 1966. Food, reproduction and organic constitution of the common antarctic echinoid *Sterechinus neumayeri* (Meissner). *Biol. Bull.,* **130**: 387–401.

Reference: *Biol. Bull.*, **159**: 737–751. (December, 1980)

THE MORPHOLOGY, LIFE HISTORY, AND SYSTEMATIC RELATIONS OF *TUBULOVESICULA PINGUIS* (LINTON, 1940) MANTER, 1947 (TREMATODA: HEMIURIDAE)[1]

HORACE W. STUNKARD

American Museum of Natural History, Central Park West at 79th Street, New York, New York; and Marine Biological Laboratory, Woods Hole, Massachusetts

ABSTRACT

Linton (1910) described *Dinurus rubeus* n. sp., a digenetic trematode from species of *Lycodontis* at Tortugas, Florida, and Manter (1931) described a second species, *Dinurus magnus,* from *Synodus foetens* at Beaufort, North Carolina. He (1947) transferred both species to the genus *Stomachicola* Yamaguti, 1934, as *Stomachicola rubea* and *Stomachicola magna.* Meanwhile, Linton (1940) described *Dinurus pinguis* from *Menidia menidia* at Woods Hole, Massachusetts, and Manter (1947) transferred this species, *pinguis,* to *Tubulovesicula* Yamaguti, 1934. Sinclair *et al.* (1972) in a 2-year study of *Stomachicola rubea* noted that the worms attain maximum size only in the stomach of "true" definitive hosts, large fishes, and that small fishes, which ingest planktonic invertebrates, serve as "transfer hosts." In these hosts, the parasites may wander about in the tissues, or may be encysted. These authors predicated that *T. pinguis* is, in such a transfer host, a stage in the life cycle of *S. rubea.* Stunkard (1973) described the development of *T. pinguis* in *M. menidia.* The worms matured without acquiring any of the essential characters of the genus *Stomachicola,* and, although the life cycles of both species were unknown, the proposal of Sinclair *et al.* was rejected. With the discovery of the life cycle and asexual generations of *T. pinguis,* described in this report, the morphological differences between *Stomachicola* and *Tubulovesicula* are supplemented with bionomic characteristics that clearly establish the validity of *T. pinguis* as a valid and independent species.

HISTORICAL BACKGROUND

Linton (1940) described *Dinurus pinguis* n. sp. from *Menidia menidia notata* taken at Woods Hole, Massachusetts. The text was supplemented by figures (Plate 9, Fig. 96 and Plate 10, Figs. 97–100). Also, specimens taken in former years from other fishes were included in the new species. Records of collection, measurements of worms, and numbers of specimens deposited in the Helminthological Collection of the U. S. National Museum were noted. The list of hosts included: (1), *Anguilla rostrata;* (2), *Roccus lineatus;* (3), *Fundulus heteroclitus;* (4), *Menidia menidia;* (5), *Synodus foetens;* (6), *Tylosurus marinus;* (7), *Menticirrhus americanus;* (8), *Merluccius bilinearis;* (9), *Menticirrhus saxatilis;* (10), *Paralichthys dentatus;* (11), *Opsanus tau;* (12), *Merulinus carolinus;* and (13), *Sphyraena borealis.*

Received June 16, 1980; accepted September 25, 1980.

[1] Investigation supported by NSF-DEB 80-06150.

The identification of worms from the stomachs of marine fishes is a precarious task. The problem becomes almost impossible when a specimen is juvenile, distorted, or partially digested. The likelihood of predation is great, since fishes eat other smaller fishes. It follows, therefore, that the report by Linton of *Dinurus pinguis* in many host species is largely irrelevant.

The species, *pinguis,* was transferred from the genus *Dinurus* Looss, 1907 to *Tubulovesicula* Yamaguti, 1934 by Manter (1947). It is a member of the subfamily Dinurinae Looss, 1907; one of 25 subfamilies listed by Yamaguti (1971) in the family Hemiuridae Lühe, 1901, an enormous taxonomic group comprising hundreds of genera. Certain authors recognize the assemblage as a superfamily, Hemiuroidea Faust, 1929 or order, Hemiurata (Markevich, 1951) Skrjabin and Guschanskaja, 1954, with the subfamilies of Yamaguti elevated to family status. Members of the group are chiefly stomach parasites of marine fishes, with a few species in freshwater fishes. The subfamily Halipeginae Esjmont, 1931 (=Halipegidae Poche, 1925) contains species parasitic in freshwater fishes, amphibians and reptiles. Indeed, Parukhin (1969) described specimens from the sea-snake, *Laticuada* sp., taken in the Gulf of Tonkin, off the coast of Vietnam, as *Tubolovesicula laticuadai* n. sp. It is the only species of Dinurinae reported from reptiles.

Yamaguti (1934) reported on parasites of fishes from the Inland Sea of Japan. In the subfamily Dinurinae he erected three new genera: *Stomachicola,* with *S. muraenesocis* n. sp., from the stomach of *Muraenesox cinereus* as type species; *Tubulovesicula,* with *T. spari* n. sp., based on a single specimen from *Sparus macrocephalus* as type; and *Magnacetabulum,* based on *M. trachuri* n. sp., from *Trachurus japonica.* Referring to the genus *Tubulovesicula,* he predicated, p. 70, "*Lecithaster lindbergi* Layman, 1930, undoubtedly belongs in this genus." In the genus he included also *Tubulovesicula anguillae* n. sp., from *Anguilla japonica* and *Tubulovesicula muraene* n. sp. from *M. cinereus.* He noted, p. 474, "This species is closely related to *Tubulovesicula anguistacauda* (Nicoll, 1915) from *M. cinereus,* but differs markedly in the size of eggs and in the posterior extent of the vesicula seminalis and pars prostatica. The uterus is confined to the body proper in young individuals, so that the differences in its posterior extent cannot be utilized for specific diagnosis." Although Yamaguti (1934) did not specifically transfer *Ectenurus angusticauda* Nicoll, 1915 to *Tubulovesicula,* the statement just quoted has that effect.

Manter (1940) described digenetic trematodes of fishes from the Galapagos Islands. In the subfamily Dinurinae, he erected two new genera: *Elytrophallus* and *Mecoderus.* The first genus was compared with the related genera, *Sterrhurus* Looss, 1907, *Tubulovesicula* Yamaguti, 1934, and *Clupenurus* Srivastava, 1935. The last genus, *Clupenurus,* he noted, should be considered a synonym of *Tubulovesicula.* The digenetic trematodes of marine fishes at Tortugas, Florida, were studied by Manter (1947). In the subfamily Dinurinae he listed the diagnostic features and recognized species of the following genera: *Lecithocladium* Lühe, 1901; *Dinurus* Looss, 1907; *Ectenurus* Looss, 1907, *Stomachicola* Yamaguti, 1934; *Tubulovesicula* Yamaguti, 1934; *Erileptus* Woolcock, 1935; *Clupenurus* Srivastava, 1935; *Elytrophallus* Manter, 1940; and *Mecoderus* Manter, 1940, *Clupenurus* Srivastava, listed as a synonym in Manter (1940), was accepted as a valid genus. In the genus *Tubulovesicula* he recognized *T. spari* Yamaguti, 1934; *T. anguillae* Yamaguti, 1934; *T. muraenesocis* Yamaguti, 1934; *T. californica* Park, 1936; *T. pseudorhombi* Yamaguti, 1938; *T. lindbergi* (Layman,

1930) Yamaguti, 1934; *T. nanaimoensis* (McFarlane, 1955) n. comb. (synonym *Dinurus nanaimoensis* McFarlane, 1935); *T. pinguis* (Linton, 1940) n. comb. (synonym *Dinurus pinguis* Linton, 1940; *T. angusticauda* (Nicoll, 1915) Yamaguti, 1934. He gave a brief diagnosis of *Stomachicola;* in addition to the type species, *S. muraenesocis* Yamaguti, 1934, he included *S. secunda* Srivastava, 1959; *S. magna* (Manter, 1931) n. comb. (synonym *Dinurus magnus* Manter, 1928); *S. rubea* (Linton, 1910) n. comb. (synonym *Dinurus rubeus* Linton, 1910).

Manter (1954) revised the genus *Tubulovesicula: T. nanaimoensis* (McFarlane, 1935) Manter, 1947 and *T. madurensis* Nigrelli, 1940 were considered synonyms of *T. lindbergi. Tubulovesicula californica* Park, 1936 was described from a single specimen found in *Enophrys bison* at Dillon Beach, California. Manter declared, p. 546, "I cannot find significant differences between this species and *T. spari* Yamaguti, 1934, *T. pseudorhombi* Yamaguti, 1934, and therefore consider them all to be *T. spari,* the type of the genus. *T. anguillae* differs only in that the ecsoma is as long as the body." This species was suppressed as a synonym of *T. spari* by Manter (1954) and as a synonym of *T. lindbergi* by McCauley (1960). *Tubulovesicula spari* was regarded as a synonym of *T. lindbergi* by Sogandares-Bernal (1959) and by Zhukov (1960). After reviewing the synonymy in *Tubulovesicula,* Sogandares-Bernal (1959, p. 104) stated, "Since *T. spari* appears to be a synonym of *T. lindbergi,* the latter becomes the type species of the genus. *T. serrani* Nagaty, 1956, which was described from one specimen, also appears to be a synonym of *T. lindbergi.* I believe that only four species of *Tubulovesicula* are valid: *T. lindbergi, T. anguisticauda, T. pinguis,* and *T. magnacetabulum.*"

Linton (1910) described *Dinurus rubeus* n. sp., from *Lycodontis moringa* and *Lycodontis funebris,* taken at the laboratory of the Carnegie Institution at Tortugas, Florida. The description was supplemented by his Figures 149–154. A second species, *Dinurus magnus,* was described by Manter (1931), based on two specimens from the stomach of the lizardfish, *Synodus foetens,* and several immature specimens from the stomach and air-bladder of the spotted trout, *Cynoscion nebulosus,* taken at Beaufort, North Carolina. Both species were transferred to *Stomachicola* Yamaguti, 1934 by Manter (1947). Corkum (1959) described gravid specimens from the stomach of *S. foetens* taken on the Mississippi Gulf Coast as *S. magnus* (Manter, 1931). He (1966) found one mature and many immature specimens of *S. magnus* in the stomach and intestines of the flounder, *Paralichtys lethostigma.* The paucity of mature and presence of a great many immature specimens suggested that the flounder is not a true definitive host, but rather one in which a few worms reach maturity while the majority remain immature. In the tonguefish, *Symplurus palgiusa,* 61 of 96 fishes had hemiurid metacercariae encysted in the body musculature. Every region of the body was involved and the encysted larvae were indicated by large, dark brown spots. The encysted worms were identified as immature specimens of *S. magnus.* Corkum concluded, p. 49, "It seems doubtful that these fishes are true intermediate hosts since Crustacea are the usual hosts for larval hemiurids." In essence, Corkum suggested that fishes serve as second intermediate hosts of *S. magnus.*

In a paper dealing with *T. pinguis,* Sinclair *et al.* (1972) predicated that this species is a stage in the life cycle of *Stomachicola rubeus* (Linton, 1910) Manter, 1947. They reported *S. rubeus* from 28 species of marine fishes taken near Sapelo Island, Georgia, between 1 October 1969 and the autumn of 1971, noting the incidence of infection and seasonal development of the parasites. They recalled that the species was described by Linton (1910) as *Dinurus rubeus* from species of *Lycodontis* at Tortugas and that Manter (1931) had described a second species, *Dinurus magnus*, from *Synodus foetens*. Both species were transferred to the genus *Stomachicola* by Manter (1947); the specific names, *rubeus* and *magnus*, were incorrectly emended to *rubea* and *magna* respectively, apparently on the mistaken belief that *Stomachicola* is a feminine name. Sinclair *et al.* (1972) suppressed *S. magnus* as a synonym of *S. rubeus* and predicated, p. 253, "an additional synonymy with *S. rubeus* is the designation *Distomum tornatum* (in part) of Linton (1940) and Dawes (1946 and as *Tubulovesicula pinguis* by Manter (1947, 1954), Skrjabin and Guschanskaja (1954), and Yamaguti (1958), Sogandares (1959), and Overstreet (1968)."

According to Sinclair *et al., S. rubeus* uses a large number of small fishes as "transfer-hosts." These individuals may belong to species which mature at a small size or are the young of larger species which presumably have similar food sources. In these hosts the parasites wander freely in the body cavity or in the tissues of various organs, often invading the liver, spleen, heart, kidney, swim-bladder, and body musculature. The worms may mature at a length of 3–3.5 mm and often leave trails of eggs as they move about. It was postulated that the transfer-hosts became infected in the spring and summer and that the worms continue to grow and mature until they are trapped in the tissues by defense reactions of the host, walled off in melanated cysts, and then "mummified" in winter and spring. Or they may be eaten by "true" definitive hosts and remain in the stomach and mature into *S. rubeus*. These authors confirmed Corkum's findings of encysted *S. magnus* in *Paralichthys lethostigma,* with heavy infections in one-third of the fishes examined. They identified the specimens as *S. rubeus* and suggested that Corkum's report of metacercariae in the tonguefish should not be interpreted literally, as it is doubtful that the organisms reported by him were metacercariae. They regarded encystment in muscular tissue as one of the variable sites selected by younger stages of the postmetacercarial specimens of this species. They noted that Corkum's account concerned only encysted worms observed during summer, thus nearly eliminating any chance of observing mature specimens in similar situations. In the tonguefish, *S. plagiusa,* Sinclair *et al.* (1972) found the worms more commonly encysted in the stomach-wall.

Sinclair *et al.* (1972) reported that *S. rubeus* develops maximum size only in "true" definitive hosts; the American eel (*Anguilla rostrata*), tarpon (*Megalops atlantica*), lizardfish (*Synodus foetens*), and kingfish (*Menticirrhus americanus*). They noted, p. 257, "The growth pattern of *Stomachicola rubea* in transfer hosts is quite diverse and depends on microhabitat. Worms encysted in the stomach wall of hosts enlarge considerably in body dimensions with little growth of the ecsoma and no apparent genital differentiation. Those free in the coelom or in body tissues elongate with comparable length of soma and ecsoma and eventually become ovigerous. Thus the diversity of forms in definitive hosts seemingly reflects the developmental stage attained in the many transfer hosts before they are ingested by a definitive host." Sinclair *et al.* traced the development of the parasite in the lizardfish (a definitive host) from June to October, with a relative increase in the length of the ecsoma as the worms grew. They reported, p. 257, "This same pattern is portrayed in coelomic inhabitants of transfer hosts, with only quite large and mature worms being found during the late fall and winter months. The larger species of transfer host appear to provide a more favorable milieu; in the kingfish and *Cynoscion* spp., the worms attain a length of 9.44–14.40 (avg. 11.48) mm in midwinter. From the pattern observed it would appear that the very largest worms (22–25 mm) of this species could well be more than 1 year of age."

Sinclair *et al.* (1972) observed that the lizardfish, *Synodus foetens*, and the kingfish, *Menticirrhus americanus*, are "true" definitive hosts of *S. rubeus*, but that young, small individuals of these species may serve as transfer hosts. They noted, p. 256, "This dual site for infection in the same host species depending on size of the host would suggest that residence in a transfer host is a necessary part of the life-cycle of *S. rubea*, as it is unlikely that the definitive hosts acquire it from the metacercarial host (most likely a small crustacean)." This statement suggests or implies a four host life cycle in *S. rubeus*. If development in a transfer host is essential before the metacercaria can mature in a definitive host, the life cycle is affected. Overstreet (1968, p. 451) reported *S. magna* (Manter, 1931) Manter, 1947 from *S. foetens* in South Florida. He noted specimens over 2 cm in length and other immature worms which appear to be *S. magna*.

Whether or not worms from *Menidia menidia*, identified as *Tubulovesicula pinguis* (Linton, 1910) Manter 1947 can continue to grow and transform into *S. rubeus* remains undetermined. The present report is a contribution toward the solution of the problem. The development of *T. pinguis* in *Menidia menidia* was described by Stunkard (1973). The morphology of the worms, taken from the body cavity and tissues of the fishes, was traced from juveniles, 0.50 mm long and 0.10 mm wide to gravid specimens 6.5 mm long and 1.50 mm wide. The ecsoma in the immediate postmetacercariae stage is merely a retractable protuberance, which elongates as the worm grows until it becomes as long as the soma in sexually mature specimens. The systematic relations of *T. pinguis* were considered in a discussion of the life cycles and classification of the hemiurid trematodes.

For more than 50 years, the mollusks of the Woods Hole region have been examined for infection by digenetic trematodes and only one hemiurid species has previously been discovered. It occurs in *Odostomia trifida* (Totten, 1834); the discovery and description of the stages in the snail were made by Hunninen and Cable (1943) and their experimental demonstration that the parasite is the asexual generation of *Lecithaster confusus* Odhner, 1905, was the first life cycle of a marine hemiurid trematode completed under controlled conditions. On 20 June 1976 a second hemiurid infection was found. It occurred in *Nassarius trivitattus* and is described in the present report as the asexual generation of *Tubulovesicula pinguis*.

MATERIALS AND METHODS

The experimental work detailed in this report was done at the Marine Biological Laboratory, Woods Hole, Massachusetts, in the summer months, 1972–1979. Since 1973, special efforts have been made to find the larval stages and complete the life cycle of *T. pinguis*. Thousands of specimens of common and even rare mollusks have been collected and examined for infection. To find the asexual generations of digenetic trematodes, snails and bivalves were isolated in bowls, 10–30, depending on size, in each bowl. On alternate days, the snails from each bowl were transferred to a bowl of fresh seawater and the water in the original bowl was examined for cercariae. If cercariae were found, the snails were isolated singly, to identify the infected one. After an isolation period of some 2 weeks, snails that had not liberated cercariae were crushed to look for immature infections.

In a collection of 110 specimens of *Nassarius trivitattatus,* made 20 June 1976, in the outer harbor at Quissett, Massachusetts, one snail was shedding cytocercous cercariae. This snail has been studied for many years; hundreds of specimens have been collected along the coast of New England, and no infection by a trematode had previously been reported. Over 500 additional specimens were taken during the summer of 1976, and no other infection was found. The infected snail continued to liberate 20–200 cercariae daily, chiefly at night, until it was crushed on 16 August to obtain the redial generations of the parasite. The tissues of the snail were intact and showed little injury as a result of the infection.

There were six large rediae in the digestive gland. One (Fig. 1) was apparently almost exhausted; it contained few developing cercariae. The others were filled with developing cercariae. No small daughter rediae were found. There were no encysted cercariae in the rediae or in the haemocoele of the snail. The cercariae did not accumulate in the haemocoele, but emerged from the snail soon after leaving the rediae. They did not encyst in the snail, but when they appeared in the seawater, the body was encased in a thin-walled cyst, to which the tail was attached. Encystment must have been rapid, almost instantaneous, and presumably occurred when the cercariae emerged from the gills of the snail into seawater. The cercariae swam vigorously at all levels in the water. There was no apparent response to light, but lashing of the tail caused the larvae to rise in the water. The cercariae were hardy; they lived for 4 days at room temperature and for 10 days at 12°C. When exhausted by swimming, the cercariae died, without excystment, on the bottom of the container.

In an attempt to complete the life cycle, the cercariae were fed to copepods, *Acartia tonsa,* which ingested them avidly.

The copepods were maintained in bowls set in running seawater in the laboratory and in a cold room at 12°C. Cultures of diatoms were provided as food. The copepods did not live well in the laboratory, although the temperature of the seawater was lower than the ambient air and some bowls were supplied with compressed air. At the reduced temperature of the cold room, the copepods lived well for 2–3 weeks. Copepods that had been fed were dissected; cercariae still in their cysts were taken from the stomachs and excysted metacercariae were found free in the body cavities of the copepods (Fig. 10). Growth was rapid in the copepods and a series of developmental stages were obtained, from recently acquired experimental to naturally acquired mature metacercariae (Fig. 11). The largest metacercariae closely resembled the smallest juveniles of *T. pinguis* and had conspicuous ecsomas (Fig. 17). The probability of specific identity was postulated, but convincing evidence was not obtained.

The rediae, cercariae, and metacercariae were studied alive, after fixation and staining with various techniques. A brief preliminary account of the asexual stages of the parasite was made (Stunkard, 1976).

In the summer of 1977, more than 600 *N. trivitattus* were collected and isolated, but no cercariae were obtained. One snail, taken on 9 June was crushed on 20 June, contained an immature infection. In the hope of obtaining cercariae later, no more snails were crushed until mid-August, but no other infection was found.

In the summer of 1978, the collection, isolation, and examination of more than 600 *N. trivitattus* yielded no infection by hemiurid trematodes. (A specimen of

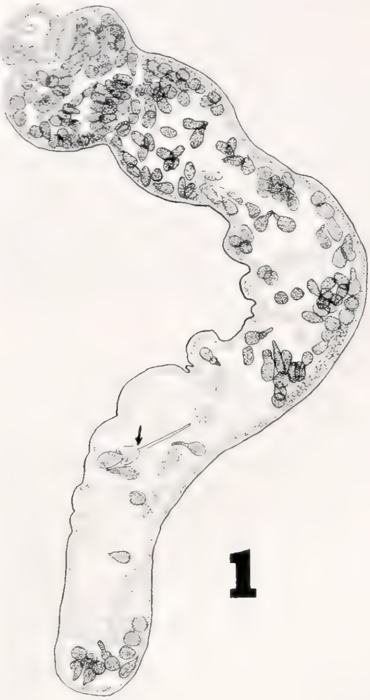
N. trivitattus, taken 20 July, was shedding a large, ophthalmotrichocercous cercaria; this was the second trematode species found in this snail. The identity and life history of the parasite were determined later; it proved to be the larva of *Lepocreadium areolatum* (Linton, 1900) described by Stunkard, 1980.) Attempts to infect *N. trivitattus* specimens by feeding them eggs of *T. pinguis* were futile. *Menidia menidia* did not appear until the middle of July and then they were small, 2–3 cm long, and their digestive tracts were filled with algae. Later, larger specimens, 3–4 cm long, had veligers and copepods in their stomachs. The first gravid worms were taken in August. Eggs of *T. pinguis* were added to bowls containing *N. trivitattus*. Examination of the snails later gave only negative results. The eggs of this species measure 0.018 by 0.012 mm, are almost transparent, and hardly visible with a dissecting microscope.

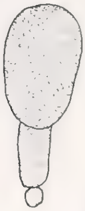
Collection, isolation, and examination of more than 600 *N. trivitattus* continued the investigation in 1979. An immature infection was disclosed in a snail crushed on 25 June (Fig. 12) and another in a snail crushed on 6 August, but no swimming cercariae were obtained. During a period of eight summers, only one snail was found shedding cystocercous cercariae, the specimen of *N. trivitattus* taken on 20 June 1976. Accordingly, another method was needed to disclose the life cycle of *T. pinguis*, and efforts were made to infect snails. In June 1979 bowls were prepared, with three snails of different sizes in each bowl. To ensure the health and vigor of these carnivorous snails, bits of such food as squid, clam, or crab tissue were provided on alternate days, before they were transferred to bowls of fresh seawater. When gravid *T. pinguis* became available, late in July, worms were placed in half tap : half seawater in watchglasses, and eggs in the terminal part of the uterus were extruded. Fresh eggs and eggs that had been embryonated in seawater at room temperature for 10 days were fed to snails that had been isolated since early June. In previous years, the eggs had been allowed to settle on algae which was added to the bowls with the snails. In the 1979 experiments, the eggs were added to minced squid, clam, or crab tissue, to increase the likelihood that they would be ingested. Eggs were fed beginning 27 July. Examination of snails began 25 August. In three snails, small spherical to oval deeply staining cellular masses (Fig. 15) were found. They are recognized as sporocysts. Larger oval sporocysts (Fig. 16) were embedded in snail tissue. Motile sporocysts were not obtained. The brief periods of summer research and the late appearance of *M. menidia* precluded consummation of the asexual generation, but experimental infection of the snail apparently completed the life cycle.

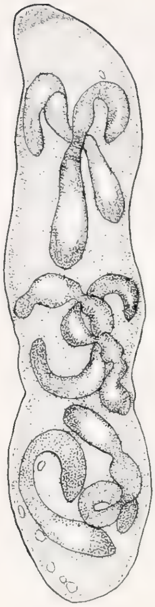
DESCRIPTIONS

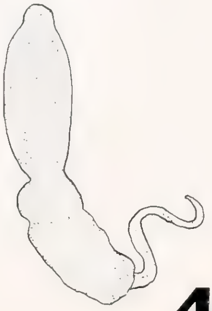
Redia (Figs. 1, 2, 12)

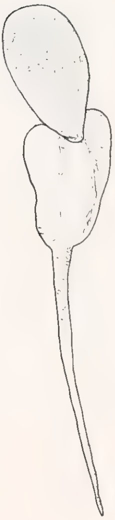
There are successive generations of rediae. Daughter rediae produce daughters, (Fig. 2), and more than 100 rediae were counted in a recent, immature infection. One of these rediae (Fig. 12) 4.00 mm long, contained thousands of germ-balls and developing cercariae. The rediae are essentially cylindrical, about 10 times as long as wide, and up to 5 mm in length. The body wall is composed of three layers. An external, syncytial tegument rests on a delicate layer of circular and longitudinal muscle fibers. Below the muscle layer is a stratum of parenchymal cells, and the lumen may be extensive in old, exhausted rediae (Fig. 1), or filled completely by developing cercariae in young rediae (Fig. 12). Small rediae are active but as they grow and the body fills with thousands of developing cercariae,


1


3


2


4


6

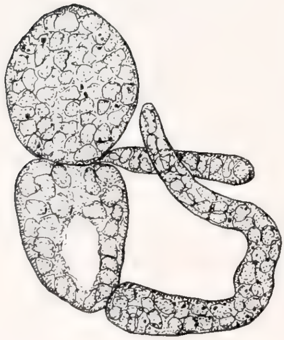

5

Figure 1. *Tubulovesicula pinguis* redia from snail collected 20 June 1976, dissected 16 August; almost exhausted; the cercaria marked with the arrow is shown in Figure 6.

Figure 2. Daughter redia, 1.14 mm long, containing six daughter rediae.

Figure 3. Young cercaria, 0.09 mm long: beginning of tripartite form.

Figure 4. Larger cercaria, 0.12 mm long.

Figure 5. Larger cercaria; body 0.077 mm, cyst 0.077 mm, tail 148 mm long.

Figure 6. Cercaria in redia (Fig. 1); body 0.045 mm, cyst 0.045 mm, tail 0.081 mm long.

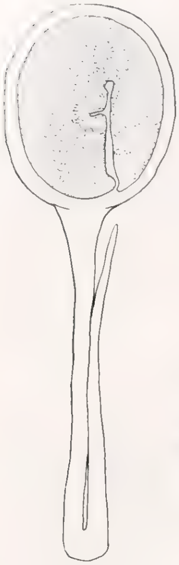
motility is restricted and large rediae slowly change shape and are unable to move. The pharynx is seldom visible in large gravid specimens, but in smaller individuals it is sometimes discernible, measuring 0.06–0.08 mm in diameter. There is a birth pore near the pharynx and a cercaria was seen to emerge tail foremost. Germ-balls first appear as small clusters of deeply staining cells, embedded in or attached to the parenchymal layer of the body wall, or free in the body cavity.

Cercariae (*Figs. 3–7*)

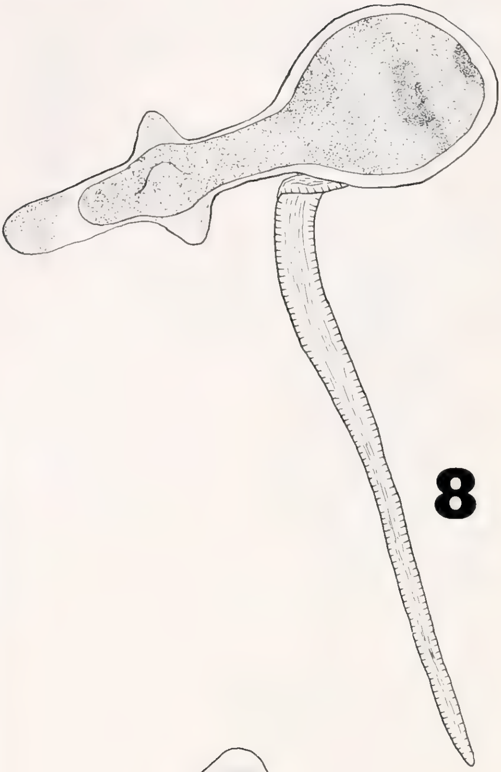
The germ-balls in the cavity of the redia are spherical to oval. As they grow, they become ovate. The narrower end elongates and two constrictions produce a tripartite form (Figs. 3–6). The larger part becomes the body of the cercaria, the medial section becomes the cyst, and the distal part becomes the tail of the cercaria. The body does not increase greatly in size; greater growth occurs in the medial and distal sections. At this stage (Fig. 6), the cercariae become motile in the redia. The cells in the body stain deeply; as development proceeds the cells in the medial section enlarge, no longer stain, and collapse, producing the cavity of the cyst and forming the delivery tube. As the cavity forms in the cyst, a strand of tissue extends from the caudal tip of the body to the tail. In encystment, it appears that this strand draws the body into the cyst, which closes over it. In the cyst, the body is bent in an inverted U-shape (Fig. 7), with the ventral side internal. The cyst is oval to pyriform, 0.05–0.055 mm in diameter with a thin-walled bulge on one side near the attachment of the tail. The tail is about 0.20 mm long and 0.015 to 0.020 mm wide at the base. A small area in the cyst between the body and tail contains what appears to be a coiled filament, but is actually the collapsed delivery tube. Fixed, stained, and mounted specimens are slightly smaller. Cercariae killed in hot water and fixed with AFAG or Duboscq-Bresil solutions have the following average measurements: diameter of the cyst, almost spherical, 0.045 mm with a conspicuous posterolateral bulge; tail 0.15 mm long and 0.012 mm wide at the base. Living specimens under coverglass pressure protruded the thin-walled posterolateral bulge of the cyst, adjacent to the attachment of the tail. A double-walled sac-like structure was everted and the body slowly passed into it (Fig. 8). The wall of the delivery tube appeared soft and flexible as the body of the cercaria emerged from the cyst and passed through the tube. In the tube wall, about 0.05–0.06 mm from the cyst, there was a ring of what appeared to be large vesicles, whose function is unclear. The delivery tube (Figs. 8, 13, 14) was about 0.20 mm long and 0.01–0.015 mm wide. Its distal tip was somewhat narrower than the rest. The tube was narrower and more constricted after the cercaria had emerged. The body was long and slender while emerging, but when free it became short and broad (Fig. 13). There is a strong tendency for the anterior end to turn ventrad (Fig. 9). Meanwhile, the tail remained attached to the empty cyst wall. Under pressure, the cyst wall sometimes ruptured, freeing the cercaria that thus did not pass through the delivery tube (Fig. 14). Fixed, stained, and mounted, emerged cercariae averaged 0.08 by 0.02 mm. The oral sucker was about 0.019 and the acetabulum, 0.022 mm in diameter.

Metacercariae

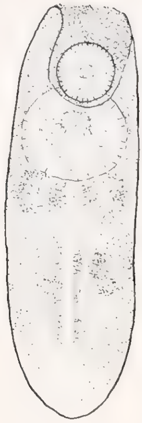
When cercariae are ingested by copepods, excystment begins in the stomach; in the intestine the delivery tube pierces the wall and ejects the cercaria into the haemocoele. In the body cavity of copepods, the metacercariae grow rapidly and specimens removed 2 weeks after ingestion (Fig. 10) measured 0.10 by 0.023 mm. During the period from July to September, 1976, dissection of copepods yielded a continuous series of developing stages from 0.10 to 0.30 or 0.40 mm in length.



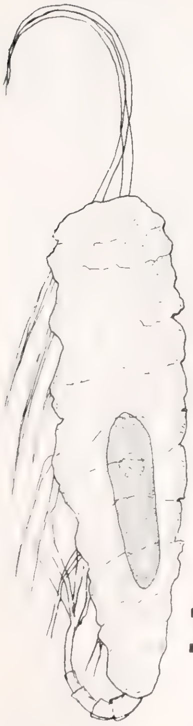
7



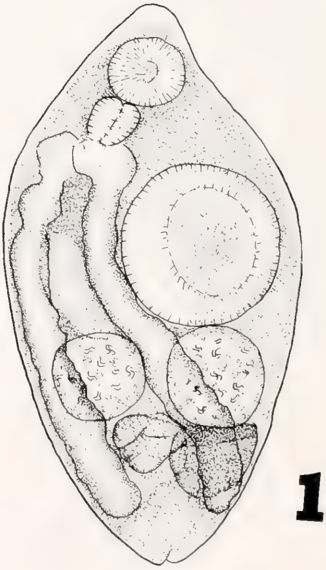
8



9



10



11

FIGURE 7. Cercaria measured alive; cyst 0.050 by 0.055 mm, tail 0.20 mm long, 0.008 wide at base. Heat killed, in alcohol; cyst 0.045 mm, tail, 16 mm long.

FIGURE 8. Cercaria, under slight pressure, beginning to emerge through the extruded delivery tube.

FIGURE 9. Cercaria after emergence: anterior end bent ventral, flattened, 0.11 by 0.035 mm.

The reproductive organs may remain in a very immature condition or the gonads may increase substantially in size. Presumably the degree of development is correlated with the time the specimen has been in the copepod. The largest metacercariae from copepods are about half as large and morphologically very similar to juveniles encysted in the stomach wall (Fig. 18) or free in the body cavity of *Menidia menidia,* as reported by Stunkard (1973). If the metacercariae are immature when ingested by *M. menidia,* they emerge from the intestine and invade the tissues where they may be encysted. If encysted, the body grows, the appendix becomes distinct, but the reproductive organs do not develop. A specimen removed from the wall of the stomach of *M. menidia* is shown in Figure 18. If they are not encysted, the metacercariae move about, invade the tissues, and eventually become mature, leaving trails of eggs. If the metacercariae are large and mature when ingested, they bore their way out of the intestine, attach themselves to the liver or one of the large veins on the wall of the intestine, begin to suck blood, and become gravid. It appears that ingestion of blood is correlated with the development of sexual maturity.

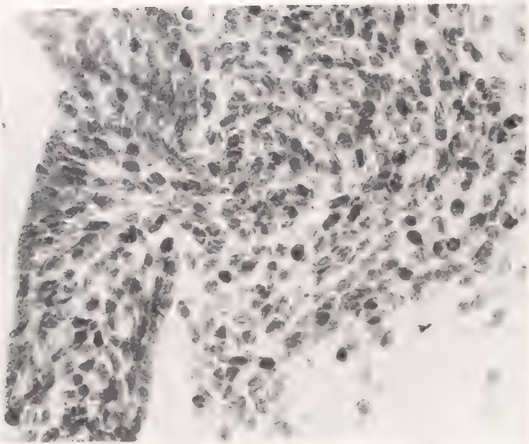
Discussion

The systematic relations of the genera *Stomachicola* and *Tubulovesicula* were called in question by the report of Sinclair *et al.*, (1972). If *T. pinguis* is merely a stage in the life cycle of *S. rubeus,* the validity and integrity of the genus *Tubulovesicula* is compromised if not undermined.

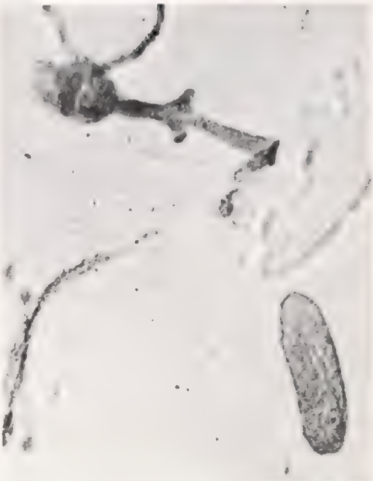
It is important to recognize that Sinclair *et al.* studied infections in many species of fishes for 2 years. They noted that the infection is seasonal, that 28 species of fish serve as transfer hosts, that they become infected in spring or early summer, and that growth is continuous. In these small fishes the worms may be encysted or may wander freely in the tissues or in the body cavity. They observed that these postmetacercarial juveniles may be killed by immune reactions of the hosts and become "mummified" in melanoid cysts. Larger piscivorous fishes apparently become infected by ingestion of "transfer hosts." In definitive hosts the worms remain in the stomach and average 11.5 mm in length by midwinter. Specimens 22–25 mm in length may be more than 1 year of age. The only "true" definitive hosts were the American eel, *Anguilla rostrata*; tarpon, *Megalops atlantica*; lizardfish, *Synodus foetens*; and kingfish, *Menticirrhus americanus.* Supporting their conception of the life cycle, Sinclair *et al.* quoted an observation of Overstreet (1968), "A low incidence of *Stomachicola* in small fish (*Synodus foetens,* lizardfish) accompanied by a higher incidence in the larger *Synodus foetens* collected at the same time, suggests that the parasite is acquired from an intermediate host not normally fed on by smaller individuals of *S. foetens*." Sinclair *et al.* added, "As well, the southern kingfish, which when small, is one of the more common transfer hosts, carries *S. rubea* in the stomach when it reaches a larger size (above 30 cm). This dual site for infection in the same host species depending on size of the host would suggest that residence in a transfer host is a necessary part of the life-cycle of *S. rubea,* as it is unlikely that the piscivorous definitive hosts acquire it from the metacercarial host (most likely a small crustacean)."

FIGURE 10. Metacercaria, 2 weeks in haemocoele of *Acartia tonsa;* 0.10 by 0.028 mm.

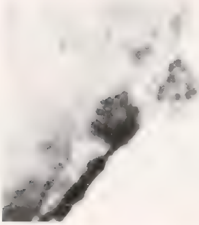
FIGURE 11. Metacercaria from *A. tonsa,* natural infection; 0.32 mm long, acetabulum 0.10 mm, oral sucker 0.032 mm, testes 0.07 mm in diameter.



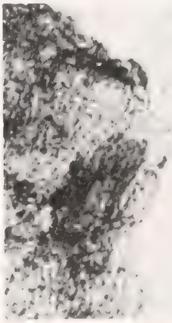
12



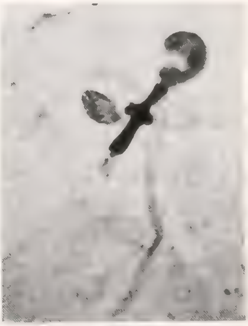
13



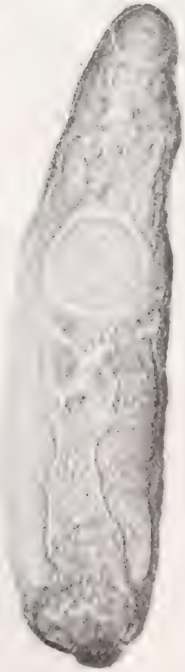
15



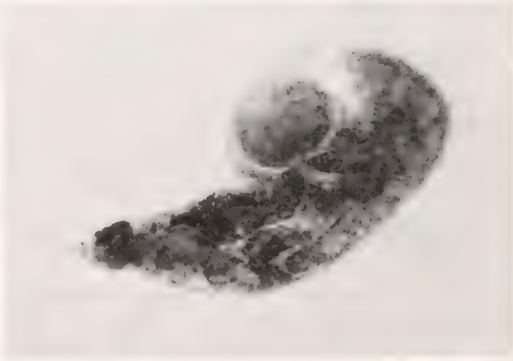
16



14



18



17

Figure 12. Redia taken 25 June 1979; body wall ruptured with emission of cercariae.
Figure 13. Empty cyst and delivery tube, and cercaria that had emerged through the tube.

There is much evidence to support the concept of Sinclair *et al.* that typically, *S. rubeus* is acquired by small transfer fishes that feed on plankton and in which it develops to a condition in which it may remain in the stomach of a larger predator. But the postulate that *T. pinguis* is a stage in the life cycle of *S. rubeus* has not been demonstrated.

The problem concerning the relationship of *Stomachicola* and *Tubulovesicula* may be resolved by a study of the comparative morphology of the two genera. Linton (1905) described specimens collected in July and August, 1901 and 1902, at Beaufort, North Carolina, as *Distomum tornatum* Rudolphi. Mature worms 8–22 mm in length were found in the stomach of *Coryphaena equisetis, Coryphaena hippurus, Menticirrhus americanus,* and *Synodus foetens,* and juvenile specimens, 6 mm long, were removed from cysts in the intestine of *Tylosurus marinus.* Linton (1910) described *Dinurus rubeus* from the stomach of *Lycodontis funebris* and *Lycodontis moringa* at Tortugas, Florida. The worms (his figs. 149–154) measured 5–25 mm in length. Manter (1931) described *Dinurus magnus* from two mature specimens (Figs. 24, 25) from the stomach of the lizardfish, *S. foetens,* and immature worms from the stomach and air bladder of spotted trout, *Cynoscion nubulosus,* taken at Beaufort, North Carolina. The adult worms were 11 and 22 mm in length and one of the juveniles was 6.5 mm long. He noted the resemblance to *D. rubeus* and included the specimens allocated by Linton (1905) to *D. tornatum* in *D. magnus.* Manter (1947) transferred *D. rubeus* and *D. magnus* to *Stomachicola.* Sinclair *et al.* (1972) suppressed *S. magnus* as a synonym of *S. rubeus. Stomachicola* is known by only two species, *S. muraenesocis* Yamaguti, 1934 from the Sea of Japan and *S. rubeus* (Linton, 1901) Manter, 1947 from the south Atlantic coast of North America. The morphology of the two species is well described and illustrated by adequate figures.

Tubulovesicula is represented by several species, although the validity of certain of them is equivocal. The morphology of the adult stage is represented by adequate descriptions of different species, including the account by Stunkard (1973) of development of *T. pinguis* in *Menidia menidia.* The discovery of the asexual generations and life cycle, described in the present report, presents additional information to support the integrity of the generic concept. The internal organization of *Stomachicola* and *Tubulovesicula* is similar. The testes are situated immediately posterior to the acetabulum; the seminal vesicle is anterior to the testes; the pars prostatica is followed by an ejaculatory duct that joins the end of the metraterm and both are included in a muscular sac that opens into the genital atrium, situated ventral to the pharynx. The ovary is post-testicular; there is a seminal receptacle but no Laurer's canal; typically there are seven (3+4) cylindrical vitelline lobes; the uterus passes posteriad for a short distance and then forward to join the metraterm.

The major differences between the genera are in overall morphology. In *Stomachicola,* the body is slender, 8–25 mm long; the acetabulum is about its diameter from the oral sucker; there is a constriction of the body in the postovarian

---

FIGURE 14. Cyst ruptured by pressure, with extrusion of delivery tube and release of cercaria, but not through the delivery tube.

FIGURE 15. Two small sporocysts, 0.059 mm in diameter, from snail fed eggs of *T. pinguis.*

FIGURE 16. Sporocyst 0.16 mm long in snail tissue, experimental infection.

FIGURE 17. Metacercarcia, natural infection from *A. tonsa,* 0.33 mm long.

FIGURE 18. Metacercaria removed from cyst in stomach wall of *Menidia menidia,* 0.50 mm long.

region and an enormous contractile tail, not a retractile ecsoma. The gonads are in the anterior one-sixth to one-fourth of the body length, and the uterine coils do not extend into the posterior half of the tail. In *Tubulovesicula*, the body is fusiform, 3–4.5 mm long; the ovary is near midbody, immediately posterior to the testes; and the uterus extends into the ecsoma which is one-fourth to one-half the body length. The most conspicuous differences between the genera are in the location of the gonads and in the elongate body and enormous tail of *Stomachicola*.

The morphological and bionomic differences between *S. rubeus* and *T. pinguis* clearly demonstrate generic distinctions and controvert the proposal that *T. pinguis* is a paratenic stage in the life cycle of *S. rubeus*.

Acknowledgment

Grateful acknowledgment is accorded Mr. Michael DiSpezio, a student in the Boston University Marine Program, for assistance in the collection and care of copepods.

LITERATURE CITED

Corkum, K. C., 1959. Some trematode parasites of fishes from the Mississippi Gulf coast. *Proc. La. Acad. Sci.*, **22**: 17–29.

Corkum, K. C., 1966. The digenetic trematodes of some flatfishes from Barataria Bay, Louisiana. *Proc. La. Acad. Sci.*, **29**: 45–51.

Hunninen, A. V., and R. M. Cable, 1943. The life-history of *Lecithaster confusus* Odhner (Trematoda: Hemiuridae). *J. Parasitol.*, **29**: 71–79.

Linton, E., 1905. Parasites of fishes of Beaufort, North Carolina. *Bull. Bur. Fish.*, **24**: 323–428. (1904).

Linton, E., 1910. Helminth fauna of the Dry Tortugas. II. Trematodes. *Carnegie Inst. Wash., Publ. No. 133. Papers Tortugas Laboratory*, **4**: 11–98.

Linton, E., 1940. Trematodes from fishes mainly from the Woods Hole region. *Proc. U. S. Nat. Museum*, **88**: 1–172

McCauley, J. E., 1960. Some hemiurid trematodes of Oregon marine fishes. *J. Parasitol.*, **46**: 84–89.

Manter, H. W., 1931. Some digenetic trematodes of marine fishes of Beaufort, North Carolina. *Parasitology*, **23**: 396–411.

Manter, H. W., 1940a. Digenetic trematodes of fishes from the Galapagos Islands and the neighboring Pacific. *Rep. Allan Hancock Pacif. Exped.*, **2(14)**: 325–497.

Manter, H. W., 1940b. The geographic distribution of digenetic trematodes of marine fishes of the tropical American Pacific. *Rep. Allan Hancock Pacif. Exped.*, **2(16)**: 531–547.

Manter, H. W., 1947. The digenetic trematodes of marine fishes of Tortugas, Florida. *Am. Midl. Nat.*, **38**: 257–416.

Manter, H. W., 1954. Some digenetic trematodes from fishes of New Zealand. *Trans. R. Soc., N. Z.*, **82(2)**: 475–568.

Parukhin, A. M., 1969. Trematode family Dinuridae Skrjabin and Guschanskaia, 1954, found in sea-snake from Tonking Bay of North Vietnam. *Uchen. Zapiski*, No. 99, *Biol. Sciences Ser.*, pp. 26–28.

Overstreet, R. M., 1968. Parasites of the inshore lizardfish, *Synodus foetens*, from south Florida, including a description of a new genus of Cestoda. *Bull. Marine Sci.*, **18**: 444–470.

Sinclair, N. R., F. G. Smith, and J. J. Sullivan, 1972. The *Stomachicola rubea: Tubulovesicola pinguis* enigma. *Proc. Helm. Soc. Wash.*, **39**: 253–258.

Skrjabin, K. I., and L. K. Guschanskaja, Eds., 1954. *Trematodes of Animals and Man*, Vol. 9. Moscow. 344 pp. (In Russian)

Sogandares-Bernal, F. 1959. Digenetic trematodes of marine fishes from the Gulf of Panama and Bimini, British West Indies. *Tulane Studies Zool.*, **7**: 69–117.

Stunkard, H. W., 1973. Observations on *Tubulovesicula pinguis* (Linton, 1910) Manter, 1947 and on the systematics of the hemiurid trematodes. *Biol. Bull.*, **145**: 607–626.

Stunkard, H. W., 1976. A new cystophorous cercaria from *Nassarius trivitattus*. *Biol. Bull.*, **151**: 433 (Abs.)

STUNKARD, H. W., 1980. The morphology, life-history, and taxonomic relations of *Lepocreadium areolatum* (Linton, 1900) Stunkard, 1969 (Trematoda: Digenea). *Biol. Bull.*, **158**: 154–163.

YAMAGUTI, S., 1934. Studies on the helminth fauna of Japan. Part 2. Trematodes of fishes. *Jpn. J. Zool.*, **5**: 249–541.

YAMAGUTI, S., 1971. *Synopsis of digenetic trematodes of vertebrates.* 2 Vols. Vol. I, 1074 pp., Vol. II. 349 plates, 1794 figs.

ZHUKOV, E. V., 1969. Endoparasitic worms of fishes from the sea of Japan and South Kevule Shoal. *Tr. Zool. Inst. Akad. Nauk SSR.*, **28**: 1–146.

Reference: *Biol. Bull.*, **159**: 752–759. (December, 1980)

GAMMA RADIATION AND HYDRANTH LONGEVITY IN CAMPANULARIA FLEXUOSA: AGE-DEPENDENCY OF DOSE-RESPONSE FUNCTION

JEROME F. WERMUTH

Department of Biology, Purdue University Calumet, Hammond, Indiana 46323

ABSTRACT

Colonies of *Campanularia flexuosa* were subjected to doses of gamma radiation of 0, 16, 24, 40, and 80 Krads. Observations were made on the longevity of hydranths relative to their age on the day of irradiation, and to the dose of radiation applied. The average life span of hydranths increased with increasing radiation dose. The increase was greater for older hydranths than for younger ones. It is suggested that the delay in hydranth regression does not approximate the aging process in higher animals. Irradiated hydranth positions do not regenerate; irradiated whole colonies eventually die. The observations on hydranth longevity are discussed in terms of radiation damage to the DNA of developing hydranths of *Campanularia flexuosa.*

INTRODUCTION

Study of the effects of ionizing radiation on growth and development of cnidarians seems to have begun with the observation by Puckett (1935) that regeneration of hydranths in colonies of *Pennaria tiarella* was suppressed by an x-ray dose of 10,000 R. Daniel and Park (1951, 1953) produced damage to the tentacles of the common brown hydra at an x-ray dose of 9600 R. Wermuth and Barnes (1967, 1968) showed an increase in stolon growth of the colonial hydroid *Campanularia flexuosa* when starting material of new colonies received an x-ray dose of 81,000 R on the fifth day after colony initiation.

Brock and Strehler (1963) and Strehler (1964) demonstrated what they considered an increase in average life span of hydranths of *Campanularia flexuosa* with x-ray doses of 500–210,000 R applied to 10-day-old colonies. This phenomenon, more properly considered as radiation-induced delay or disruption of the normal regression-replacement cycle of hydranths of this species, was confirmed by Wermuth (1968) for x-irradiation, and by Wermuth and Barnes (1973, 1975) for gamma-irradiated *Campanularia flexuosa* hydranths. Wermuth and Barnes (1975) monitored several other post-irradiation growth functions in this species: average amount of new stolon material added per colony, average number of new hydranth positions added to colony starting material, average number of new uprights added to new stolons, and average number of hydranth positions added to uprights of new stolons. All decreased with increasing gamma-radiation doses. Implicit but not stated in any of the above papers is the deleterious effect of ionizing radiation on entire colonies of *Campanularia flexuosa.* The author of this paper found that doses greater than 16 Krad resulted in colony death, even though hydranth regression was delayed.

Received August 20, 1979; accepted August 19, 1980.

Campanularia flexuosa hydranths normally undergo a regression-replacement cycle (Crowell, 1953) whose duration is a function (among other things) of the temperature at which the colonies are raised. Generally, the warmer the temperature, the shorter the length of time between the initiation of a hydranth and its regression, followed by regeneration of a new hydranth at that position.

Studies by Brock and Strehler (1963) and Strehler (1964) represent dose-response studies on a single growth function in *Campanularia flexuosa*, but the data were not presented as dose-response curves. These studies did not consider the effect that the age of the hydranth may have on its susceptibility to delayed regression with increasing dosage of x-irradiation. The present paper shows that age at the time of irradiation does affect the increase in delay of regression. The data also suggest that the increase in delay of hydranth regression may well be the result of interference with several biological operations.

MATERIALS AND METHODS

Experimental colonies of *Campanularia flexuosa* were initiated as described in Wermuth and Barnes (1969). Stock colonies from which experimental colonies were derived were obtained from Dr. Sears Crowell, Department of Biology, Indiana University, Bloomington, Indiana. The stock colonies (strains E and Q) used by the author were originally collected by Dr. Crowell and Dr. Charles Wyttenbach near Woods Hole, Massachusetts.

Each experimental group consisted of 15 colonies. Three colonies were cultured on single microscope slides, with five slides per experimental group. Each group of five slides was kept in a 10-slide tissue-staining rack. Groups of colonies were maintained at constant temperature in Instant Ocean medium either in a 20 gal Instant Ocean aquarium or in 200 ml tissue-staining dishes in a Hot Pack environmental chamber on a shaker table. The medium of colonies maintained in tissue-staining dishes was replaced every other day. Colonies were fed twice daily by placing the staining racks in a beaker containing freshly hatched brine shrimp (*Artemia*) nauplii. The nauplii were filtered and washed once in Instant Ocean medium before resuspension in fresh Instant Ocean medium for feeding purposes. Hydranth developmental stages were observed daily. The ability to capture nauplii was used to determine whether a polyp was a mature hydranth, or had not regressed. This ability was determined in doubtful cases by gently squirting a stream of brine shrimp nauplii past the polyps' tentacles. Capture of at least one brine shrimp by a tentacle was considered sufficient to determine a polyp as a mature non-regressed hydranth.

No attempt was made to select equal sized groups of hydranths for statistical purposes. All irradiated hydranths were included in the study. Data presented in Figure 1, and Tables I, II, and III were collected only from polyps produced by new stolon growth from starting material. Data from starter material polyps are not included. To equalize ages, a hydranth was considered to have an age of +1 days if it acquired the ability to feed on the day of irradiation. A polyp of age −2 days did not become a feeding hydranth until 2 days after the day of irradiation. A polyp of +3 days began feeding 3 days before the day of irradiation. Thus, the data contained in Figure 1, and Tables I, II, and III are reported as age in days, as mature polyp, on the day of irradiation.

Gamma irradiation of colonies was accomplished by placing the colonies and tissue staining racks in tissue staining dishes containing 200 ml of fresh Instant

TABLE I

Hydranth number, average life span, and standard deviation of average life span by hydranth age on day of irradiation and gamma radiation dose.

Group by radiation dose	Hydranth age in days as mature polyps on day of irradiation											
	−6	−5	−4	−3	−2	−1	+1	+2	+3	+4	+5	+6
0 Krad												
Number of hydranths	0	7	10	3	0	2	5	1	3	0	0	1
Average life span, days	—	11.1	12.0	12.3	—	—	13.6	14.0	13.6	—	—	14
Standard deviation	—	±0.3	±0.6	±0.6	—	±0	±0.5	—	±2.1	—	—	—
16 Krad												
Number of hydranths	3	6	12	4	6	6	2	7	4	1	0	0
Average life span, days	12.0	12.7	11.8	12.5	13.0	13.2	12.5	13.9	14.0	15.0	—	—
Standard deviation	±0	±0.8	±0.9	±0.6	±0.6	±0.7	±0.5	±1.6	±0	—	—	—
24 Krad												
Number of hydranths	2	7	4	3	9	3	4	4	3	1	0	0
Average life span, days	11.5	11.1	13.7	14.3	15.1	17.3	16.7	19.3	18.7	18.0	—	—
Standard deviation	±0.7	±1.1	±0.4	±0.6	±1.4	±1.5	±2.2	±1.0	±2.5	—	—	—
40 Krad												
Number of hydranths	0	8	5	2	6	4	4	3	3	1	0	0
Average life span, days	—	13.9	14.2	16.0	16.8	18.3	20.7	19.7	20.0	20.0	—	—
Standard deviation	—	±1.1	±1.1	±0	±1.0	±1.7	±1.3	±1.2	±2.0	—	—	—
80 Krad												
Number of hydranths	3	5	3	3	3	3	1	4	4	0	0	0
Average life span, days	15.7	16 4	17.0	20.0	20.7	21.0	17.0	22.0	24.5	—	—	—
Standard deviation	±1.5	±2.7	±1.0	±0	±0.6	±1.7	—	±0.8	±0.8	—	—	—

Ocean medium, and placing the tissue staining dishes at set distance from a Co^{60} source. The colony-containing slides were moved to the five slide positions in the tissue staining rack closest to the radiation source for the period of irradiation, and were moved to the other rack positions post-irradiation. The radiation source consisted of a series of Co^{60} rods which could be raised or lowered from a well located in the center of the floor of the radiation room, which was approximately 4 × 4 m. Colonies to be irradiated were placed on the floor adjoining the source rods; control colonies were kept in the adjoining control room, from which the experimenter could raise or lower Co^{60} rods by remote control. Radiation was delivered in a single 80 min dose. The temperature of the Instant Ocean medium surrounding non-irradiated control colonies rose no more than 1°–2°C during the course of the irradiation; the temperature of the medium surrounding irradiated colonies rose no more than 2°–3°C during that same time. Immediately post-irradiation, all colonies were returned to the aquarium or to the environmental chamber. Colonies returned to the chamber were placed in fresh Instant Ocean medium. Gamma-radiation dosage was monitored by the HE-6 dosimetry method (McLaughlin *et al.*, 1971). Vials of the dosimetry reagent were placed alongside the staining racks containing the colony-bearing slides at the level of the slides.

RESULTS

The following data were recorded from a cloned strain (designated *E*) of *Campanularia flexuosa*. All experimental colonies were derived from stock

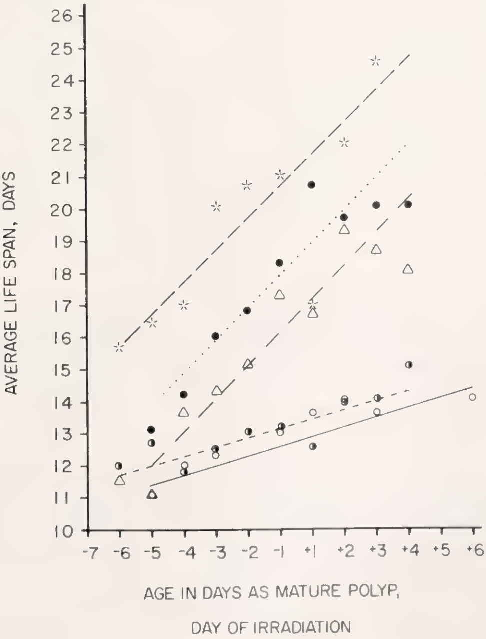


FIGURE 1. Average life span in days of hydranths of *Campanularia flexuosa* as a function of age on day irradiated and gamma radiation dose. Symbols: open circle = non-irradiated controls; half closed circle = 16 Krad dose; open triangle = 24 Krad dose; closed circle = 40 Krad dose; star = 80 Krad dose. See Table I for standard deviations and number of hydranths at each point.

Table II

Analysis of covariance test for significant differences between slopes of curves for hydranth longevity as a function of hydranth age on day of irradiation and radiation dose. Values are the probability that the observed differences between group slopes are due only to chance (COVARY program of SPSS—See text).

Group by radiation dose	0 Krad	16 Krad	24 Krad	40 Krad
16 Krad	0.5803	—		
24 Krad	0.0000	0.0000		
40 Krad	0.0000	0.0000	0.7865	
80 Krad	0.0000	0.0000	0.8033	0.9917

colonies produced from an original parent colony by asexual propagation. Similar results were obtained from another parent colony strain (designated Q).

Table I presents data on the average life span of hydranths of *Campanularia flexuosa* by age on day of irradiation for colonies receiving 0, 16, 24, 40, and 80 Krad of gamma radiation 11 days after initiation of the colonies. Figure 1 is a graphic representation of the data contained in Table I. Each point represents the average life span of hydranths of that age class on the day of irradiation for a particular radiation dose. Standard deviations and the number of hydranths included in each age class at a particular radiation dose are presented in Table I. First-order linear regression curves in Figure 1 were computed using the COVARY program of the Statistical Package for the Social Sciences (SPSS), as were values for slopes, intercepts, and statistically significant differences between them. For all five curves, increasing hydranth longevity with increasing age relative to the day of irradiation is indicated, as is an increase in the intercepts. Curves for colonies receiving 24, 40, and 80 Krad of gamma radiation have a greater positive slope than the curves for colonies receiving 0 and 16 Krad of gamma radiation.

Tables II and III show results of statistical analysis of differences among the slopes and intercepts of the curves for hydranth longevity at each radiation dose.

Discussion

Colonies of *Campanularia flexuosa* seemingly present an opportunity to study one developmental process that resembles aging in higher animals. The hydranths undergo a regression-replacement cycle (Crowell, 1953) in which new polyps are produced at specific sites on the colonial organism's uprights (facilitating record

Table III

Analysis of covariance test for significant differences between intercepts of curves for hydranth longevity as a function of hydranth age on day of irradiation and radiation dose. Values are the probability the differences between group intercepts are due only to chance (COVARY program of SPSS—See text).

Group by radiation dose	0 Krad	16 Krad	24 Krad	40 Krad
16 Krad	0.0985	—	—	—
24 Krad	0.0000	0.0000	—	—
40 Krad	0.0000	0.0000	0.0000	—
80 Krad	0.0000	0.0000	0.0000	0.0000

keeping), undergo development with morphologically distinct stages, and become hydranths having the appropriate feeding structures. Eventually the hydranths regress by autolysis (Brock, 1970). Cellular material is absorbed into the upright and the empty calyx is cast or drops off. Within a day or so a new hydranth is regenerated at the same spot and passes through the same developmental stages. The number of regression-replacement cycles possible at a position may be limitless, though the time required for a cycle is longer at lower than at higher temperatures.

Colonies of this species may be valuable in aging studies because they seem not to age. Occasionally large colonial mats form on the sides of the aquaria where stock colonies are maintained. Gross observations of the mats detect no signs of deterioration from a central point, such as one would expect if older portions of the colonies aged and died. Large portions of these stolonic mats have been scraped away and examined microscopically. Such examination reveals that few stolon or upright perisarc tubes are devoid of coenosarc. Colonies of the E strain of *Campanularia flexuosa* have been grown in continuous culture and asexually propagated by the author since 1970. If aging involves a functional decline in the later years of an animal's life span (Lamb, 1977), then aging in *Campanularia flexuosa* colonies has not been observed or reported.

Hydranths, which are parts of colonies, do show a functional decline with time. Individual hydranths lose their capacity to capture, ingest, and digest brine shrimp. They autolyze and disappear. In that sense individual hydranths have a life span or a measurable longevity. Brock and Strehler (1963), Strehler (1964), Wermuth (1968), and Wermuth and Barnes (1973, 1975) all demonstrated increasing hydranth longevity with increasing x- and gamma-radiation doses. The data presented here suggest at least two types of effect of gamma radiation on hydranth longevity as a function of hydranth age on day of irradiation. One effect is an increase in the longevity of all hydranths, regardless of age, with increasing radiation dose. The other is an increase in the longevity of specific hydranth age classes, shown by the significant increase in slope between (but not within) the combination of non-irradiated control and 16-Krad irradiated hydranths on the one hand, and 24-, 40-, and 80-Krad irradiated hydranths on the other. In graphic terms, there is both rotation and translation of the curves due to the gamma radiation. Whether these two very different effects are caused by the radiation at two different "sites" or are pleiotropic remains to be seen.

Most likely, however, the aspects of hydranth longevity considered here should not be viewed within the framework of aging. Hydranths are parts of animals. Their autolysis resembles that of tail absorption in tadpole metamorphosis. Developmentally, the frog proceeds to bigger and better; *Campanularia flexuosa* simply regenerates a new hydranth. The radiation effects on various parts of this species have been previously described (Wermuth and Barnes, 1975). Even though heavy doses of ionizing radiation increase functional persistance of hydranths, these doses do not delay regression. They seem to stop it. Altman *et al.* (1970) have stated that the primary radiation damage to cellular systems is damage to the cellular DNA. Heavy radiation doses probably interfere with the differential gene expression which generates the regression-replacement cycle of hydranths such that the hydranths do not regress at all but disintegrate as the entire colony dies. One can only speculate within the framework proposed by Altman *et al.* (1970) as to why irradiated newer hydranths are less persistent than their further developed counterparts. The normal regression-replacement cycle with its periodic destruction and replacement of parts does not lead to the death of the colony but may well have evolved to maintain the colony as a non-senescent animal.

One puzzling aspect of the data presented in Figure 1 and Table I is the increase in the life span of control hydranths with increasing age; that is, the slope of the curve for non-irradiated control hydranths is not a line parallel to the abscissa of the graph in Figure 1. This phenomenon has been observed in the hydranths of other young colonies of *Campanularia flexuosa*. It is not known whether the same is true for older, well established colonies. Deterioration in culture conditions leading to earlier regression of younger hydranths is not considered likely, since the total amount of biomass in the aquarium was small compared to the total volume of the aquarium. In addition, the total increase in biomass during an experiment was small compared to the total biomass in the aquarium during the experiment, since heavily overgrown stock colonies were always maintained in the aquarium at the same time. But possible interaction between the 15 colonies on five slides within the volume of a single slide rack, leading to the earlier regression of younger hydranths, is not ruled out, though such a strategy for a colonial organism with many feeding parts seems self defeating.

ACKNOWLEDGMENTS

I wish to thank Dr. C. D. Barnes, formerly of the Department of Life Sciences, Indiana State University, and Dr. John M. Emlen of the Department of Biology, Indiana University, for their help. Much of this work was performed while the author was a visiting research professor and a National Cancer Institute special research fellow (#FO3 C14 53590) at Indiana State University.

LITERATURE CITED

ALTMAN, K. I., G. GERBER, AND S. OKADA, 1970. *Radiation Biochemistry. Volume I: Cells.* Academic Press, New York and London. 366 pp.

BROCK, M. A., 1970. Ultrastructural studies on the life cycle of a short lived metazoan, *Campanularia flexuosa*. II. Structure of an old adult. *J. Ultrastruct. Res.*, **32**: 118–141.

BROCK, M. A., AND B. L. STREHLER, 1963. Studies on the comparative physiology of aging. IV. Age and mortality of some marine cnidaria in the laboratory. *J. Gerontol.*, **18**: 23–28.

CROWELL, P. S., 1953. The regression-replacement cycles of hydranths of *Obelia* and *Campanularia*. *Physiol. Zool.*, **25**: 319–327.

DANIEL, G. E., AND H. D. PARK, 1951. The effects of x-ray treated media on *Hydra* tentacles. *J. Cell. Comp. Physiol.*, **38**: 417–425.

DANIEL, G. E., AND H. D. PARK, 1953. Glutathione and x-ray injury in *Hydra* and *Paramecium*. *J. Cell. Comp. Physiol.*, **43**: 359–368.

LAMB, M. J., 1977. Biology of Ageing. Blackie and Son Limited, Glasgow. 184 pp.

MCLAUGHLIN, W. L., E. K. HUSSMANN, H. H. EISENLOHR, AND L. CHALKEY, 1971. A chemical dosimeter for monitoring gamma-radiation doses of 1–100 Krad. *Int. J .Appl. Radiat. Isot.*, **22**: 135–140.

PUCKETT, W. O., 1935. The effects of x-irradiation on the regeneration of the hydroid *Pennaria tiarella*. *Anat. Rec.*, **64**: 30.

STREHLER, B. L., 1964. Studies on the comparative physiology of aging. III. Effects of x-irradiation dosage on age-specific mortality rates of *Drosophila melanogaster* and *Campanularia flexuosa*. *J. Gerontol.*, **19**: 83–87.

WERMUTH, J. F., 1968. The effects of x-irradiation on stolon growth and hydranth production in *Campanularia flexuosa*; glutathione as a radio-protective agent. Ph.D. thesis. Indiana University. 81 pp.

WERMUTH, J. F., AND C. D. BARNES, 1967. The effects of x-irradiation on stolon growth in *Campanularia flexuosa*. *J. Gen. Physiol.*, **50**: 2505.

WERMUTH, J. F., AND C. D. BARNES, 1968. Differential glutathione protection of radiation effects at two different sites in *Campanularia flexuosa*. *Radiat. Res.*, **35**: 496.

WERMUTH, J. F., AND C. D. BARNES, 1969. Differential radio-protection by glutathione of two growth functions in the hydroid *Campanularia flexuosa*. *Biol. Bull.*, **137**: 375–383.

WERMUTH, J. F., AND C. D. BARNES, 1973. The effects of gamma radiation on several growth functions in the hydroid *Campanularia flexuosa*. *Radiat. Res.*, **55**: 561–562.

WERMUTH, J. F., AND C. D. BARNES, 1975. Dose-response effects of gamma radiation on several growth functions of *Campanularia flexuosa*. *Biol. Bull.*, **148**: 344–356.

Reference: *Biol. Bull.*, **159**: 760–772. (December, 1980)

MANDIBULAR GLAND OF THE BLUE CRAB, *CALLINECTES SAPIDUS*

ASHLEY I. YUDIN,[1,3] RICHARD A. DIENER,[3,4] W. H. CLARK, JR.,[1,3]
AND ERNEST S. CHANG[1,2]

[1] *Department of Animal Science, University of California, Davis, California 95616;*
[2] *Bodega Marine Laboratory, University of California, Bodega Bay, California 94923;*
and [3] *National Marine Fisheries Service, Galveston, Texas 77550*

ABSTRACT

This study examines the mandibular glands of *Callinectes sapidus* in both males and females. These pale yellow organs are located at the anterior end of the paired chitinous tendons that extend from the mandibles to the dorsal carapace. Channels of hemolymph subdivide the glands into cords. Each cord is composed of irregularly shaped cells with eccentric nuclei. The karyoplasm contains patches of condensed chromatin closely associated with the nuclear envelope. The most distinguishing feature of mandibular gland cells is two distinct types of smooth endoplasmic reticulum. Golgi complexes, mitochondria, and lipidlike inclusions are also prominent cytoplasmic organelles. The plasma membrane, when adjacent to hemolymph channels, exhibits varying degrees of convolution. These structural features are characteristic of other steroid-producing cells. Although mandibular glands do not store or secrete ecdysteroids, implants of the glands do accelerate molting in shrimp.

INTRODUCTION

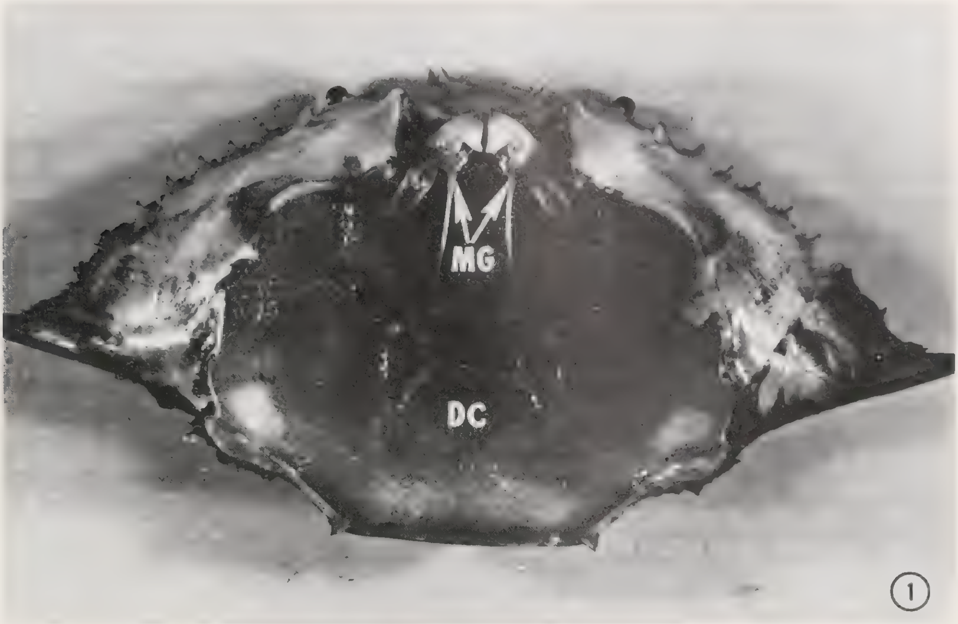
Alternating periods of growth and molting in crustaceans have been a subject of interest for many years. Hormonal control of ecdysis was first postulated at the turn of the century, with observations of precocious molting after eyestalk removal (Zeleny, 1905; Megusar, 1912). Later work shows that the X-organ sinus gland complex, located in the eyestalks, produces a molt-inhibiting hormone that regulates ecdysis (Brown and Cunningham, 1939; Carlisle, 1953). It has been proposed that the level of molt-inhibiting hormone in the hemolymph controls the Y-organ (Passano and Jyssum, 1963), a site of ecdysteroid production (Chang and O'Connor, 1977).

LeRoux (1968) describes another pair of crustacean glands that he calls mandibular organs. These glands are reported to be unnecessary for normal ecdysis (Sochasky *et al.*, 1972). Others report, however, that the mandibular glands of male intermolt crabs conform to the general morphological features of an endocrine gland (Hinsch and Al Hajj, 1975) and show fine structural changes after eyestalk ablation (Hinsch, 1977). Furthermore, the mandibular glands of lobsters, shrimp, and crayfish also change histologically and in fine structure during the molt cycle

Received May 5, 1980; accepted Sept. 25, 1980.

Abbreviations: Radioimmunoassay, RIA; Smooth endoplasmic reticulum, SER; tubular endoplasmic reticulum, TER.

[4] Present address: Bureau of Land Management, P.O. Box 6770, Albuquerque, New Mexico 87107.



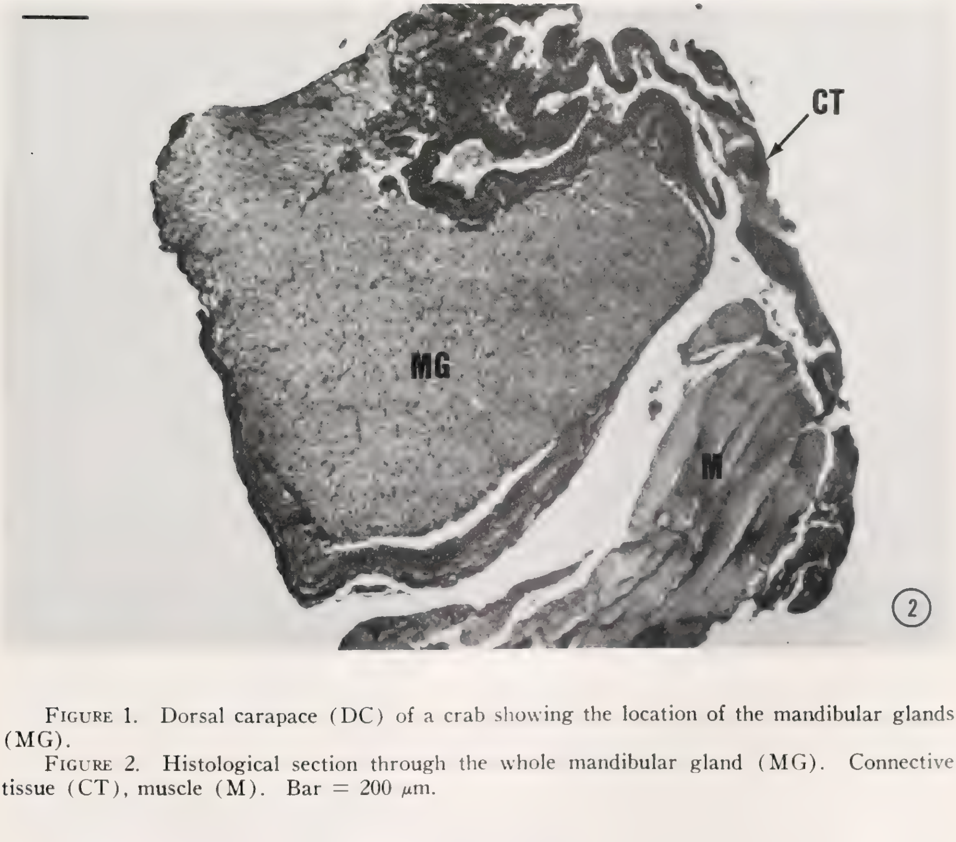
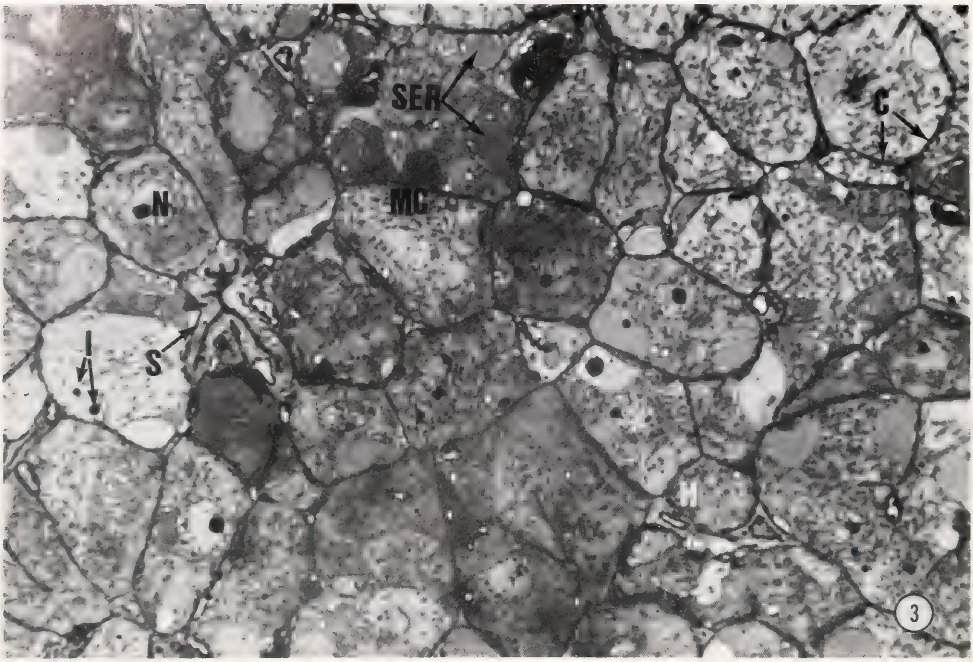
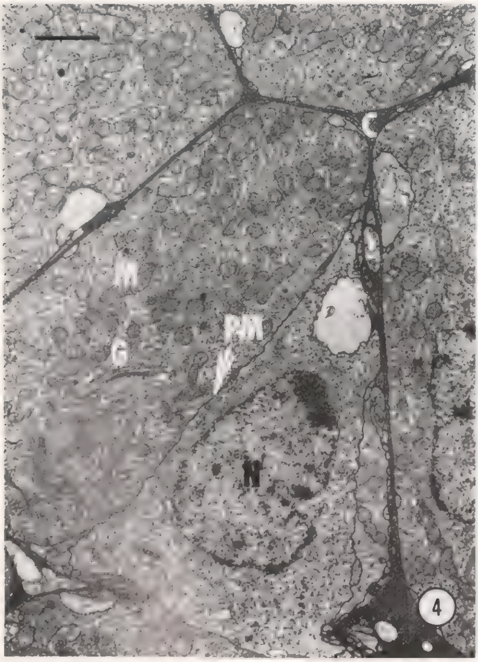


FIGURE 1. Dorsal carapace (DC) of a crab showing the location of the mandibular glands (MG).

FIGURE 2. Histological section through the whole mandibular gland (MG). Connective tissue (CT), muscle (M). Bar = 200 μm.





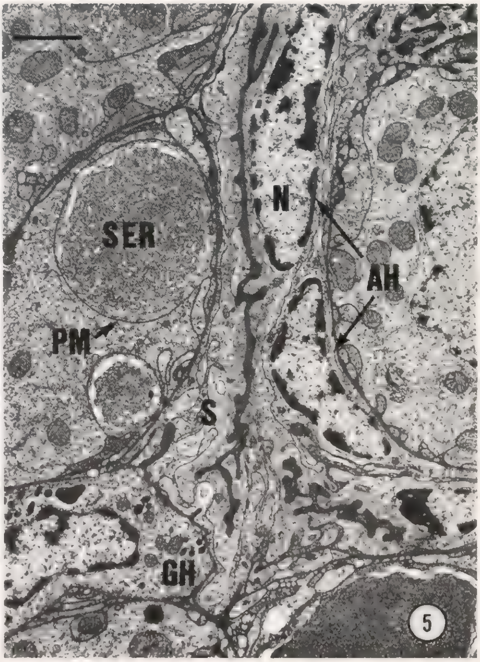


FIGURE 3. Light micrograph revealing the cord arrangement of the mandibular cells (MC). Nucleus (N), hemocyte (H), hemolymph channel (C), smooth endoplasmic reticulum (SER), hemolymph sinus (S), lipidlike inclusions (I). Bar = 11 μm.

FIGURE 4. Electron micrograph illustrating the gland's cellular arrangement. Nucleus (N), Golgi complexes (G), mitochondria (M), hemolymph channel (C), plasma membrane (PM). Bar = 0.73 μm.

FIGURE 5. Section through a hemolymph sinus (S). Nucleus (N), agranular endoplasmic reticulum (SER), agranular hemocytes (AH), granular hemocytes (GH), plasma membrane (PM). Bar = 0.9 μm.

(Aoto *et al.*, 1974; Byard *et al.*, 1975). To understand the mandibular gland and its possible roles, we initiated an anatomical and cytological description of these glands from male and female crabs during various stages of the molt cycle. A bioassay was designed to ascertain the glands' possible physiological role, and a radioimmunoassay (RIA) was used to test for the presence or secretion of ecdysteroids.

MATERIALS AND METHODS

Blue crabs were supplied from South Carolina Marine Resources Institute and purchased throughout the year from bait dealers in Galveston, Texas, with the most abundant supplies obtained from April to September. They were maintained in 350-gal tanks and fed scraps of fish, squid, and shrimp.

Glands were excised from males and nonovigerous females. Tissues for histochemical examinations were fixed in seawater-buffered formalin, dehydrated in a graded alcohol series, and cleared in chloroform for 15 min. These tissues were then infiltrated with paraplast, sectioned at 7 μm, and stained with periodic acid-Schiff reagent (Lillie, 1965).

Glands used in light and electron microscopic studies were fixed in Karnovsky's (1965) solution for 1 hr and post-fixed in phosphate-buffered 1% osmium tetroxide for 30 min. They were then dehydrated in a graded acetone series, embedded in a low-viscosity epoxy resin (Spurr, 1969), and sectioned with glass and diamond knives on a Sorvall MT-2B ultramicrotome. The sections were mounted on uncoated copper grids, stained with uranyl acetate (Venable and Coggeshall, 1965) and lead citrate (Watson, 1958), and viewed with an Hitachi HS-8 electron microscope. Thick plastic sections for light microscopy were stained with 1% methylene blue buffered in a 1% sodium borate solution.

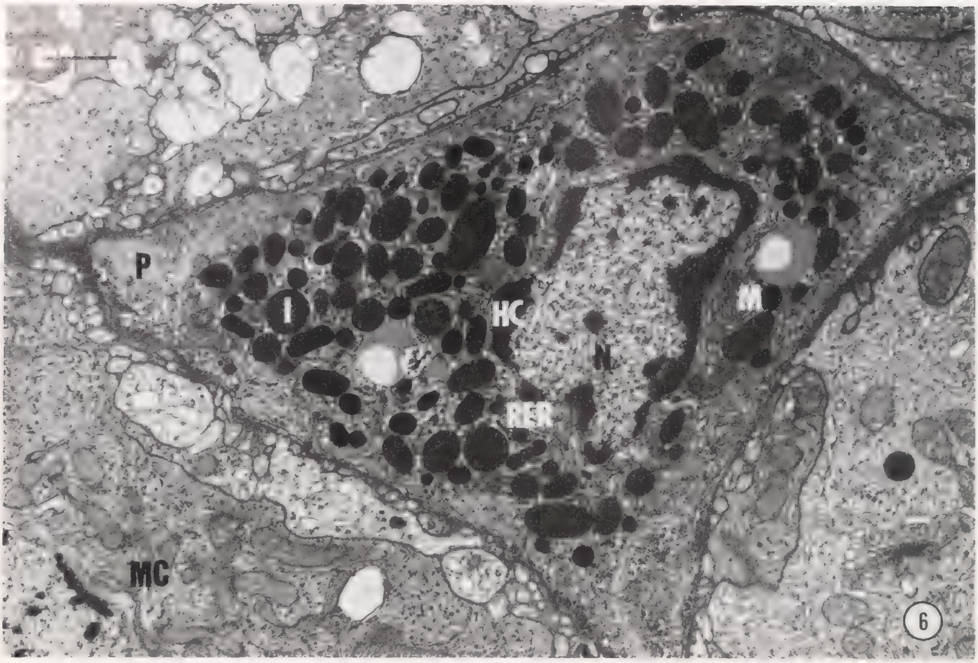
To determine the effects of mandibular gland implants on ecdysis, bioassays were performed on intermolt white shrimp, *Penaeus setiferus*. Shrimp were used because of their relatively short molt cycle. Four separate groups were tested to note the length of time between molts: control, which received no treatments; sham control that received jabs with a sterile needle; sham control that received implants of crab muscle; and experimental, which received mandibular gland implants. Each group of shrimp was held in a separate 20-gal aquarium and fed a commercially prepared food. The control group was left unmolested, but the sham and experimental animals received either jabs or implants in the abdominal region twice a week.

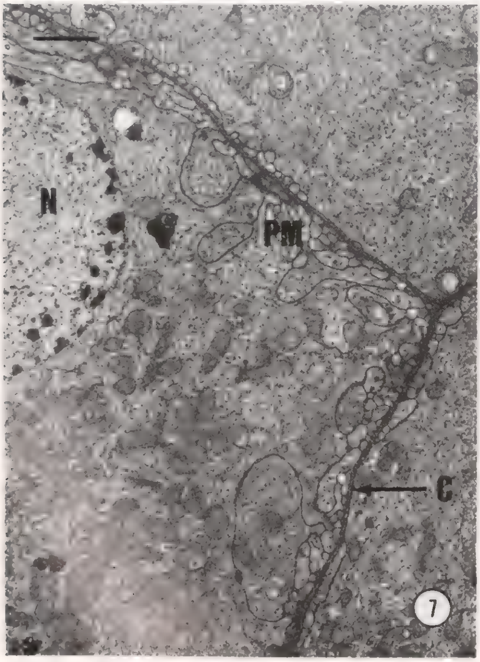
The RIAs for ecdysteroids were performed as previously described (Chang and O'Connor, 1979) on extracts of mandibular glands and Y-organs from *C. sapidus*. The antiserum used was that of Borst and O'Connor (1974), in which the antigen was the carboxymethyloxime of 20-hydroxyecdysone conjugated to bovine serum albumin. The radiolabeled ligand was ^{3}H-ecdysone (specific activity of approximately 68 Ci/mmol). Cultures consisted of either single Y-organs and mandibular glands or one of each from intermolt crabs, in 200 μl of saline (Pantin, 1934). After 1 day of culture at 23°C under an ambient atmosphere, 25 μl aliquots were removed from the culture wells (Costar 3524) and dried before RIA. In addition, freshly explanted mandibular glands were extracted with 200 μl of methanol. After centrifugation, the supernatants were removed, reduced in volume under a stream of nitrogen, and subjected to RIA.

RESULTS

Microscopic analysis of the glands

Gross Morphology. Mandibular glands from the blue crab *C. sapidus* are pale yellow, somewhat spherical, and approximately 1–2 mm in diameter. They are





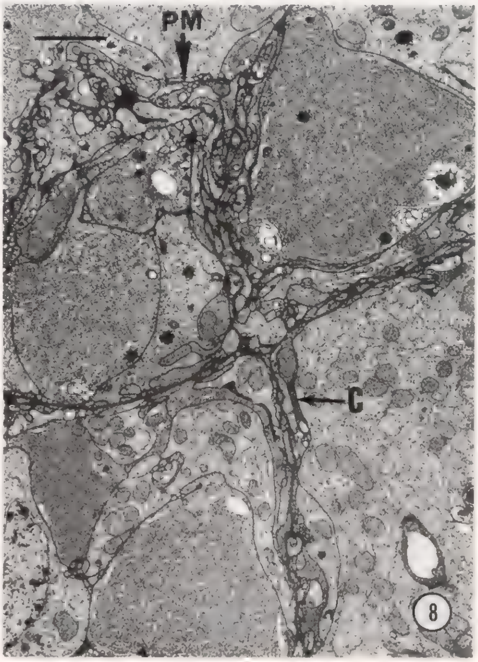
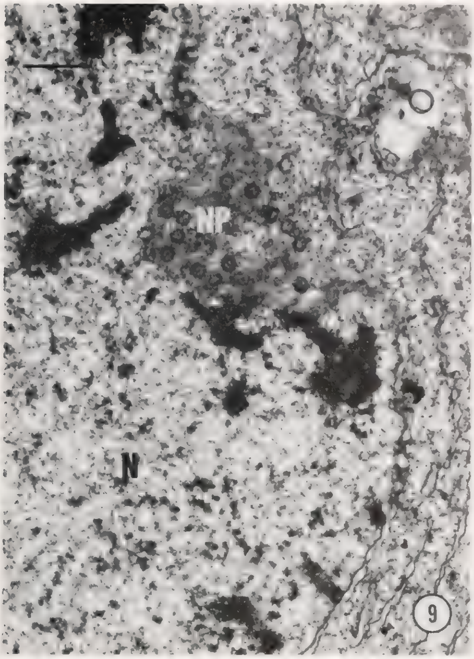
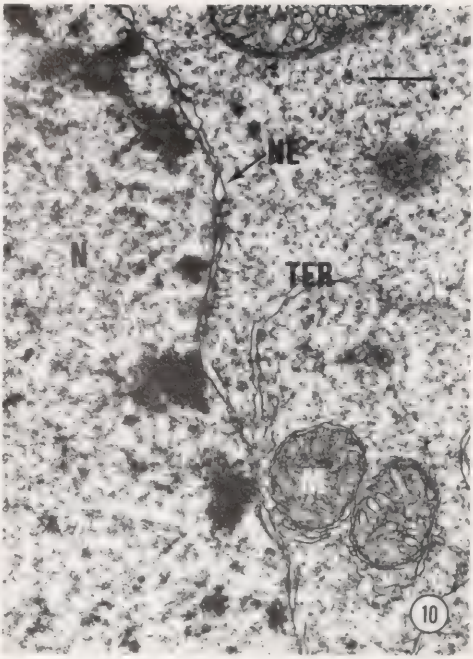


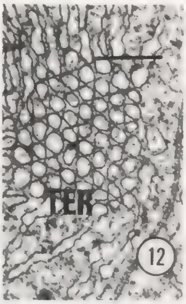
FIGURE 6. Electron micrograph of an intercellular granular hemocyte. Pseudopodia (P), nucleus (N), heterochromatin (HC), rough endoplasmic reticulum (RER), mitochondria (M), lipidlike inclusions (I), mandibular cells (MC). Bar = 0.57 μm.

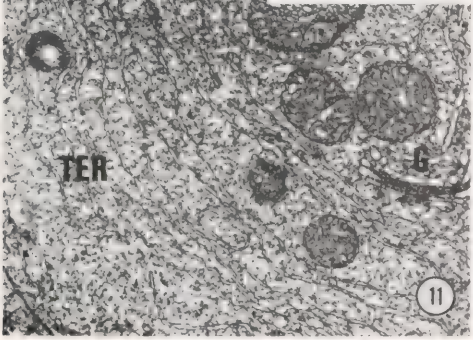
FIGURE 7. Plasma membrane (PM) convolution of mandibular gland cells adjacent to hemolymph channels (C). Nucleus (N). Bar = 0.78 μm.

FIGURE 8. Micrograph of extensive plasma membrane (PM) convolution. Hemolymph channel (C). Bar = 1.4 μm.









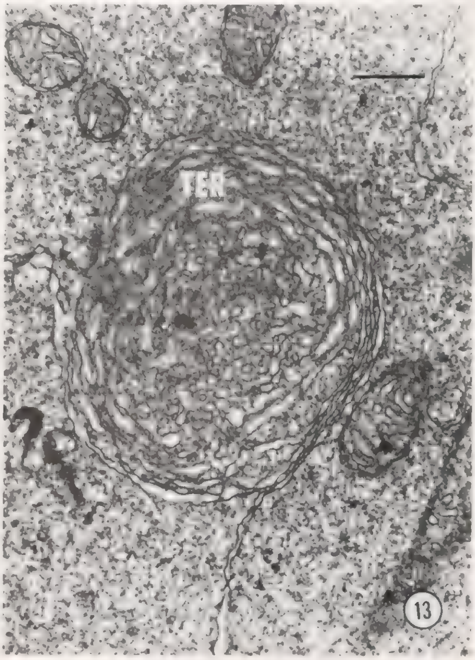


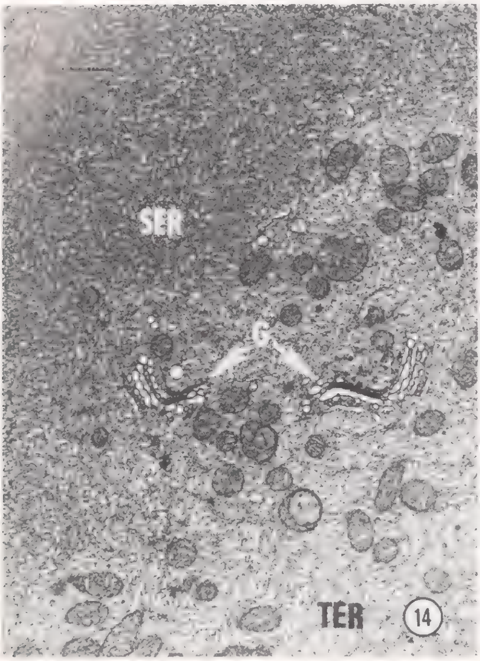
FIGURE 9. Array of nuclear pores (NP) along the nuclear envelope. Nucleus (N). Bar = 0.24 μm.

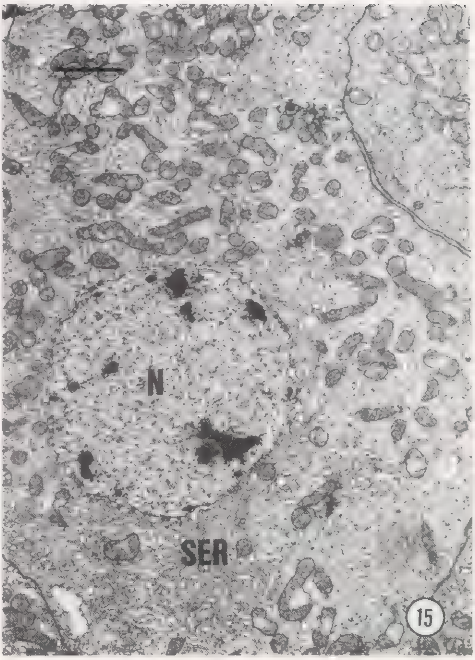
FIGURE 10. Agranular endoplasmic reticulum (TER) branching off the nuclear envelope (NE) and extending into the cytoplasm. Nucleus (N), mitochondria (M). Bar = 0.21 μm.

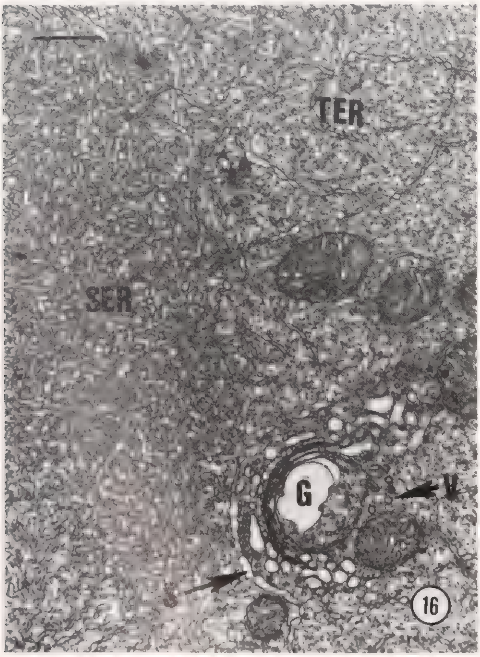
FIGURE 11. Stacked tubular endoplasmic reticulum (TER) within the cytoplasm. Golgi complex (G). Bar = 0.37 μm.

FIGURE 12. Cross-section through TER revealing its tubular configuration. Bar = 0.13 μm.

FIGURE 13. Whorl of TER. Bar = 0.26 μm.







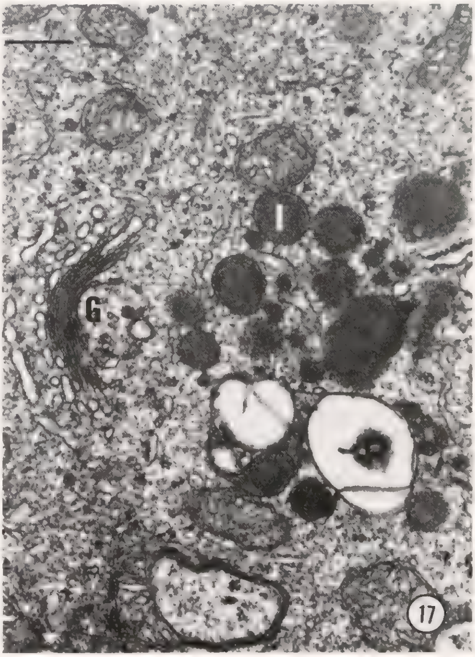


FIGURE 14. A micrograph showing a large aggregate of SER in association with Golgi complexes (G). Branches of TER can be seen along the periphery of the SER and throughout the cytoplasm. Bar = 0.9 μm.

FIGURE 15. A relatively nonactive mandibular cell with only small patches of SER. Nucleus (N). Bar = 0.86 μm.

directly posterior to the mandibles and closely associated with the anterior portion of the paired chitinous tendons that extend from the mandibles to the dorsal carapace (Fig. 1). Layers of connective and muscle tissue surround and firmly attach the glands to the tendons (Fig. 2). The glands' size does not vary significantly with the sex of the crab.

Light Microscopy. The general architectural features of the mandibular glands are easily observed by light microscopy (Fig. 3). Intercellular hemolymph channels subdivide the glands into numerous cords of cells. The mandibular cells vary considerably in shape and tend to have eccentric nuclei with condensed heterochromatin around the peripheral karyoplasm (Fig. 3). Cytoplasmic vacuolation is not pronounced, but dark-staining inclusions and large amorphous accumulations of smooth endoplasmic reticulum are evident (Fig. 3).

Fine Structure. When viewed at the fine structural level, the cord configuration of the mandibular gland is clearly demonstrated (Fig. 4): As also seen by light microscopy, each cell within a cord is directly opposed to intercellular hemolymph at some point, and the hemolymph channels are interconnected with hemolymph sinuses.

Within the sinuses are two distinct types of hemocytes, granular and agranular (Fig. 5). Granular hemocytes are also observed outside the sinuses and surrounded by mandibular gland cells (Fig. 6). In all granular hemocytes, the nuclei are irregularly shaped, with condensed chromatin around the peripheral karyoplasm, and the few mitochondria are approximately 0.1–0.2 μm in diameter. Dispersed throughout the cytoplasm are dense lipidlike inclusions and cisternae of rough endoplasmic reticulum. The pseudopodial projections are free of cytoplasmic organelles and vary in shape. More lipidlike inclusions are seen in granular hemocytes outside the sinuses than in those within the sinuses. In contrast, agranular hemocytes are restricted to the sinuses and lack cytoplasmic inclusions.

The plasma membrane of mandibular gland cells is convoluted where it is adjacent to hemolymph channels (Fig. 7). The extent of this convolution can be correlated to the types of organelles within gland cells (Fig. 4, 7, 8). Gland cells that lack or show few agranular endoplasmic reticula, Golgi complexes, enlarged mitochondria, and lipidlike inclusions, have only minimal plasma membrane convolution (Fig. 4). Gland cells with an extensive network of agranular endoplasmic reticulum and one or more of the other organelles exhibit elaborate plasma membrane convolutions (Fig. 8). The presence, absence, or degree of proliferation of these organelles within gland cells varies substantially among crabs but remains relatively uniform within a single gland.

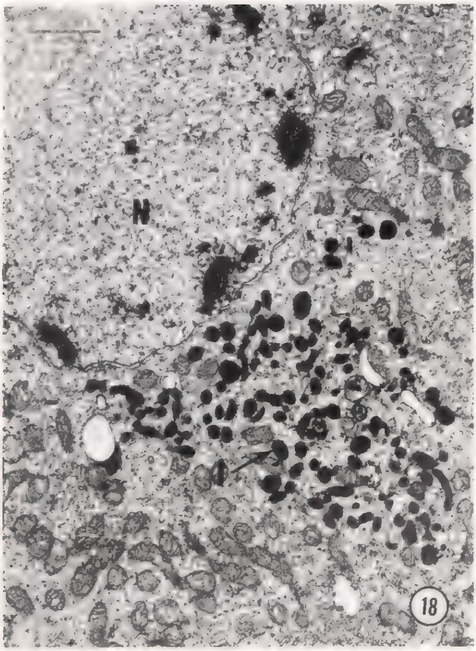
Regardless of cytoplasmic variability, the nuclei of gland cells are relatively consistent. They contain numerous nuclear pores and patches of condensed heterochromatin along the peripheral nucleoplasm (Fig. 9). Commonly, branches of agranular endoplasmic reticulum extend from the nuclear envelope into the cytoplasm (Fig. 10).

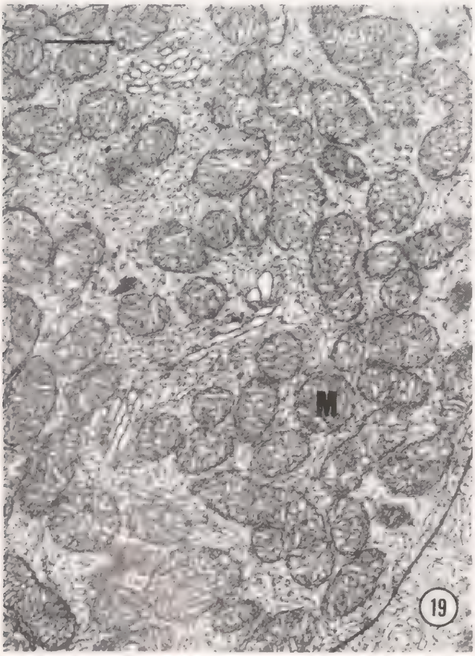
A prominent feature of mandibular gland cells is their two structurally distinct types of agranular endoplasmic reticulum. To distinguish between these two, the anastomosing reticulum will be referred to as smooth endoplasmic reticulum (SER),

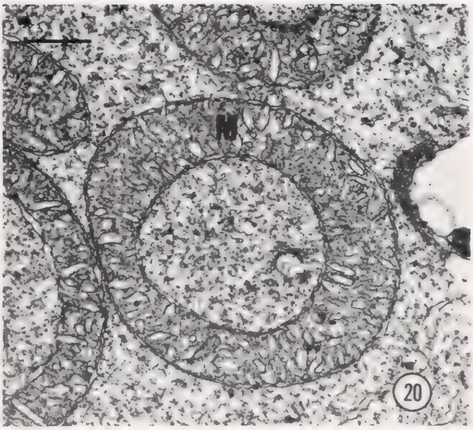
---

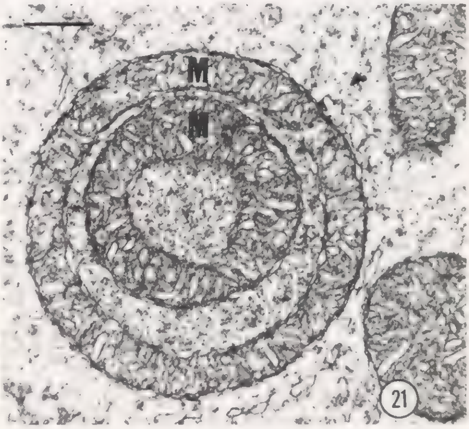
FIGURE 16. A higher magnification showing more clearly the association of SER with TER and Golgi complexes (G). Vesicles (V), saccules (S). Bar = 0.5 μm.

FIGURE 17. A micrograph showing the occasional close association of Golgi complexes (G) with lipidlike inclusions (I). Bar = 0.23 μm.









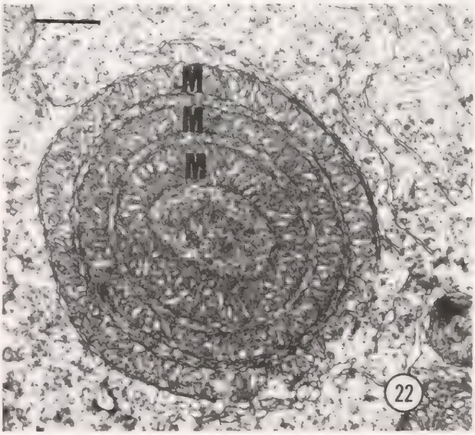


FIGURE 18. A large accumulation of lipidlike inclusions (I) within the cell's cytoplasm. Nucleus (N). Bar = 0.68 μm.

FIGURE 19. A population of typical mitochondria (M). Bar = 0.41 μm.

FIGURE 20. A ring-shaped mitochondrion (M). Bar = 0.25 μm.

FIGURE 21. Concentric rings of mitochondria (M). Bar = 0.35 μm.

FIGURE 22. Triple concentric rings of mitochondria (M). Bar = 0.35 μm.

and the second type, which has a definite tubular configuration, will be referred to as tubular endoplasmic reticulum (TER).

The TER extends from the nuclear envelope, as mentioned previously, to form three structurally different configurations: broken fragments, tightly coiled whorls (Figs. 8, 13), and a series of stacked reticular membranes in parallel arrays. The tubular nature of the membranes is clearly revealed when viewed in cross section (Figs. 11, 12). In gland cells that have considerable amounts of SER, fragmented TER is commonly found in association with or near the SER accumulations, but neither tightly coiled whorls nor stacked arrays of TER are found in such cells (Figs. 14, 16).

The variable appearance of SER is another distinguishing feature of mandibular gland cells. Glands differ dramatically in the amount they contain. Small, isolated bundles of SER often are dispersed throughout the cytoplasm (Fig. 15). Occasionally, elaborate networks of SER occupy a large portion of the cell's cytoplasm (Figs. 8, 14).

In mandibular cells, Golgi complexes are frequently observed in close association with large accumulations of SER (Fig. 14, 16). In such association, the convex surface of the Golgi complexes appears to produce saccules, and the concave surface releases vesicles (Fig. 16). Golgi complexes are also dispersed throughout the cytoplasm, often with dense, lipidlike inclusions near their concave surfaces (Fig. 17). These inclusions usually are scattered throughout the cytoplasm. Occasionally, however, they are grouped within a confined area (Fig. 18).

Another feature of the cytoplasm of mandibular gland cells is the variety of mitochondrial shapes. Most conform to the typical rod, oval, or circular shapes (Fig. 19), but some cells contain occasional large, ring-shaped mitochondria (Fig. 20) with one to three concentric rings (Figs. 21, 22). All of the mitochondria have tubular cristae oriented perpendicularly to their long axes.

Bioassay

Table I gives results of tissue implants used to examine the possible hormonal role of mandibular glands: Time between molts decreased in shrimp injected with implants of mandibular gland tissue.

TABLE I

The effect of mandibular gland implants on the molt cycle of white shrimp (P. setiferus). *Control 1: unmolested; Control 2: jabbed with needle; Control 3: injected with implants of crab muscle; Experimental: injected with mandibular gland implants from male crabs (31.89 days between molts) and from female crabs (33.11 days between molts).*

	Control 1	Control 2	*C. sapidus* tissue implants	
			Control 3	Experimental
Number of shrimp	11	11	20	26
Number of shrimp × length of experiment	788	765	847	1127
Number of molts	9	10	12	35
Mean length of time between molts (days)	87.56	76.50	70.58	32.20

Organ culture

Following 1 day of incubation in saline, [RIA] of the media in which either Y-organs or mandibular glands had been cultured revealed the mean RIA activity of four Y-organ cultures to be 47.3 pg/μl and that of four mandibular gland cultures to be negligible (4 pg/μl). Co-culture of the Y-organ and a mandibular gland from the same animal did not increase *in vitro* accumulation of RIA activity after 2 days in culture over that of the contralateral Y-organ cultured alone. Analysis of the methanolic extracts of freshly explanted mandibular glands revealed that these glands do not store RIA-active material.

Discussion

The mandibular gland cells described in the present study conformed closely to the criterion for active steroid production, in contrast to the glands previously described (Hinsch and Al Hajj, 1975; Bazin, 1976). This criterion is proliferation of characteristic ultrastructural features of steroid-secretion cells: extensive agranular endoplasmic reticulum, prominent Golgi complexes, mitochondria with variable sizes and shapes, and lipid droplets (Christensen and Gillim, 1969).

In this study, the concurrent appearance of two distinct types of agranular endoplasmic reticulum, SER and TER, was the most distinguishing feature of the mandibular gland cells. The degree of SER proliferation differed greatly among glands, as it does in steroid-producing cells of vertebrates (Christensen and Gillim, 1969; Loft, 1972) and other invertebrates (Locke, 1969). The TER of the gland cells exhibited three distinct configurations: stacked, whorled, and fragmented. The stacked configurations are associated with steroid production in testicular tissue of *Plucrodeles waltle* (Picheral, 1968), and the whorls are observed in the corpora allata of insects (Joly *et al.*, 1968) and the corpus luteum of rats (Enders and Lyons, 1964). The appearance and variable configuration of both SER and TER within different cells of the same gland suggest the synthesis of more than one type of glandular product.

Previously, Long and Jones (1967) postulated that Golgi complexes are the site of steroid conjugation. In mandibular gland cells, the Golgi complexes' significance in steroid production was indicated by their membranous association with SER and their production of saccules and vesicles. In addition, the concave surfaces of the Golgi complexes were closely opposed to the lipidlike inclusions, which may be steroidal.

Another cytological feature of mandibular gland cells was the variable size and shape of mitochondria. Such variations are also described for the prothoracic gland of silkworm larvae during the molt cycle (Beaulaton, 1968). Similar ring-shaped mitochondria are also observed in mandibular gland cells from crabs following eyestalk removal (Hinsch, 1977; Bazin, 1976). These large concentric rings of mitochondria may be responsible for cleavage of the cholesterol side chain, as occurs in mammals (Christensen and Gillim, 1969).

Although the fine structural descriptions showed that the mandibular gland is steroidal in nature, the exact physiological function of this organ is still unclear. Results of several studies, however, suggest some role of the mandibular gland in the molt cycle. Comparative fine-structural studies of the mandibular gland and Y-organ in macrurans reveal changes in activity during the molt cycle (Aoto *et al.*, 1974; Byard *et al.*, 1975). The Y-organ appears to change most dramatically during late intermolt, whereas the mandibular glands increase in activity during premolt (Aoto *et al.*, 1974; Byard *et al.*, 1975). After injections of the proposed molting hormone, 20-hydroxyecdysone, the mandibular glands of crayfish develop extensive networks of agranular endoplasmic reticulum, modified mitochondria, and giant granules (Miyawaki and Taketomi, 1970). Because the Y-organ is the source of ecdysteroid (Chang and O'Connor, 1977) and the mandibular glands appear to show activity soon after Y-organ activity, there may be a relationship among the Y-organ, the mandibular glands, and ecdysis.

Implanting mandibular glands into shrimp decreased time between molts, and implantation into crabs accelerated proecdysis (Connell, 1970). If the mandibular gland does promote molting, it does not seem to do so by storing or secreting ecdysteroid, as indicated by the lack of significant amounts of RIA-active material in the medium of the cultured glands. These results are similar to those obtained in crayfish following molt induction (Keller and Schmid, 1979). In addition, our co-culture experiments indicated that the mandibular glands did not synthesize an ecdysone precursor or trophic hormone to stimulate production of ecdysone by the Y-organ.

The mandibular gland, then, must synthesize a steroid other than ecdysone to fulfill its apparent endocrine function. The fine structural results of this study have led to several testable explanations of the mandibular gland's function. Perhaps the ecdysone produced by the Y-organ affects the activity of the mandibular gland, which, in turn, may influence ecdysis. Another possibility is that the substance produced by the mandibular gland is involved in the reproductive cycle, which is closely related to ecdysis.

ACKNOWLEDGMENTS

We thank Ms. D. Revera and Ms. M. Bruce for their technical assistance; Dr. P. Talbot, Dr. F. Conte, and Dr. E. Couch for their help in reviewing the manuscript; and Ms. A. McGuire for editorial assistance in preparing the manuscript. The ecdysteroid antiserum and radiolabeled ecdysone were generous gifts from Dr. J. D. O'Connor.

LITERATURE CITED

AOTO, T., Y. KAMIGUCHI, AND S. HISANO, 1974. Histological and ultrastructural studies on the Y-organ and mandibular organ of the freshwater prawn, *Palaemon paucidens*, with special reference to their relation with the molting cycle. *J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool.*, **19**: 295–306.

BAZIN, F., 1976. Mise en évidence des caratères cytologiques des glandes stéroïdogènes dans les glandes mandibulaires et les glandes Y du Crabe *Carcinus maenas* (L) normal et épédonculé. *C.R. Hebd. Acad. Sci. Ser. D. Sci. Nat.*, **282**: 739–741.

BEAULATON, J., 1968. Modification ultrastructurales des cellules sécrétrices de la glande prothoracique de vers à soie au cours des deux derniers âges larvaires. *J. Cell Biol.*, **39**: 501–525.

BORST, D. W., AND J. D. O'CONNOR, 1974. Arthropod molting hormone: Radioimmunoassay. *Science*, **178**: 418–419.

BROWN, F. A., JR., AND O. CUNNINGHAM, 1939. Influence of the sinus gland of crustaceans on normal viability and ecdysis. *Biol. Bull.*, **77**: 104–114.

BYARD, E H., R. R. SHIVERS, AND D. E. AIKEN, 1975. The mandibular organ of the lobster, *Homarus americanus*. *Cell Tissue Res.*, **162**: 13–22.

CARLISLE, D. B., 1953. Studies on *Lysmata seticaudata* Risso. VI. Notes on the structure of the neurosecretory system of the eyestalk. *Pubbl. Stn. Zool. Napoli*, **24**: 435–447.

CHANG, E. S., AND J. D. O'CONNOR, 1977. Secretion of α-ecdysone by crab Y-organs *in vitro*. *Proc. Nat. Acad. Sci. USA.*, **74**: 615–618.

CHANG, E. S., AND J. D. O'CONNOR, 1979. Arthropod molting hormones. Pp. 797–814 in B. M. Jaffe and H. R. Behrman, Eds., *Methods of Hormone Radioimmunoassay*. Academic Press, New York.

CHANG, E. S., B. A. SAGE, AND J. D. O'CONNOR, 1976. The qualitative and quantitative determinations of ecdysones in tissues of the crab, *Pachygrapsus crassipes*, following molt induction. *Gen. Comp. Endocrinol.*, **30**: 21–33.

CHRISTENSEN, A. K., AND S. W. GILLIM, 1969. The correlation of fine structure and function in steroid secreting cells, with emphasis on those of the gonads. Pp. 415–488 in K. W. McKerns, Ed., *The Glands*. Appleton-Century Crofts, New York.

CONNELL, P. A. M., 1970. The hormonal control of molting in the dwarf crayfish, *Cambarellus shufeldti*. Ph.D. Thesis, Dept. of Biology, Tulane University, New Orleans, La. 160 pp., #71-8043.

ENDERS, A. C., AND W. R. LYONS, 1964. Observations on the fine structure of lutein cells. II. The effect of hypophysectomy and mammotrophic hormone in the rat. *J. Cell Biol.*, **22**: 127–141.

HINSCH, G. W., 1977. Fine structural changes in the mandibular gland of the male spider crab. *Libinia emarginata* (L.) following eyestalk ablation. *J. Morphol.*, **154**: 307–316.

HINSCH, G. W., AND H. AL HAJJ, 1975. The ecdysial gland of the spider crab, *Libinia emarginata*. *J. Morphol.*, **145**: 179–188.

JOLY, L., P. JOLY, A. PORTE, AND A. GIRARDIE, 1968. Étude physiologique et ultrastructurale des corpora allata de *Locusta migratoria* L. (Orthoptère) en phase grégaire. *Arch. Zool. Exp. Gen.*, **109**: 703–728.

KARNOVSKY, M. J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.*, **27**: 137a.

KELLER, R., AND SCHMID, E., 1979. *In vitro* secretion of ecdysteroids by Y-organs and lack of secretion by mandibular organs of the crayfish following molt induction. *J. Comp. Physiol.*, **130**: 47–353.

LEROUX, A., 1968. Description d'organes mandibulaires nouveaux chez les Crustacés Decapodés. *C. R. Hebd. Acad. Sci., Ser. D. Sci. Nat.*, **266**: 1414–1417.

LILLIE, R. D., 1965. *Histopathological Techniques and Practical Histochemistry*, McGraw-Hill, New York, p. 199.

LOCKE, M., 1969. The ultrastructure of the oenocytes in the molt/intermolt cycle of an insect. *Tissue Cell*, **1**: 103–154.

LOFT, B., 1972. The sertoli cell. *Gen. Comp. Endocrinol. Suppl.*, **3**: 636–648.

LONG, J. A., AND A. L. JONES, 1967. The fine structure of the zona glomerulosa and zona fasiculata of the adrenal cortex of the opossum. *Am. J. Anat.*, **120**: 463–471.

MEGUSAR, F., 1912. Experimente uber den Farbwechsel der Crustaceen. *Arch. Ent. Mech. Org.*, **33**: 462–665.

MIYAWAKI, M., AND Y. TAKETOMI, 1970. Structural changes induced in the cells of the Y gland of crayfish by the administration of ecdysterone. *Zool. Mag. (Tokyo)*, **79**: 150–155.

PANTIN, C. F. A., 1934. On the excitation of crustacean muscle. *J. Exp. Biol.*, **11**: 11–27.

PASSANO, L. M., 1960. Molting and its control. Pp. 473–536 in T. H. Waterman, Ed., *The Physiology of Crustacea*, Vol. 1, Academic Press, New York.

PASSANO, L. M., AND S. JYSSUM, 1963. The role of the Y-organ in crab proecdysis and limb regeneration. *Comp. Biochem. Physiol.*, **9**: 195–213.

PICHERAL, B., 1968. Ultrastructure des cellules du tissu glandulaire du testicule de *Pleurodeles waltle* Micah. *J. Microsc. (Paris)* **7**: 115–134.

SOCHASKY, J. B., D. E. AIKEN, AND N. H. F. WATSON, 1972. Y-organ, molting gland and mandibular organ: a problem in decapod Crustacea. *Can. J. Zool.*, **50**: 993–997.

SPURR, A. E., 1969. A low viscosity epoxy-resin embedding medium for electron microscopy. *J. Ultrastruct. Res.*, **26**: 31–43.

VENABLE, J. H., AND R. COGGESHALL, 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.*, **25**: 407.

WATSON, M. L., 1958. Staining of tissues for electron microscopy with heavy metals. *J. Biophys. Biochem. Cytol.*, **4**: 475.

ZELENY, C.,, 1905. Compensatory regulation. *J. Exp. Zool.*, **2**: 1–102.

INDEX

A

Aber, J. D., see J. P. Schimel, 464
Abstracts of papers presented at the general scientific meetings of the Marine Biological Laboratory, August 21–23, 1980, 441
Acartia tonsa, formation of siliceous teeth, 349
Acrorhagial response in sea anemones, 117
Actin, 442
Adar, Orit, see M. Rahat, 376
Adelman, William J., Jr. *et al.*, Squid axon sodium channels are blocked reversibly by aphantoxin derived from a prokaryotic algae, 478
  see Alan J. Hodge, 471
Adeyomo, Oyewole *et al.*, Change in cyclic nucleotide content during germinal vesicle breakdown in *Spisula* oocytes, 466
ADP ribosylation in nuclei isolated from different embryonic stages of *Arbacia*, 474
Aeolidia papillosa (slug), 480
Age determination in the keyhole limpet *Fissurella crassa* Lamarck (Archaeogastropoda: Fissurellidae) based on shell growth rings, 606
Aggressive behavior among sea anemones, 117
Aging, in *Campanularia flexuosa*, 752
Alberte, R. S., see S. B. Brown, 499
  et al., Colorless proteins in phycobilisomes of rhodophycean and cyanobacterial species, 498
  see D. E. Kirchman, 461
  see T. A. Kursar, 500
Aldridge, David W., *et al.* Effects of temperature acclimation on nitrogen metabolism in two littorinid snails, 447
  see W. D. Russell-Hunter, 452
Alexander, David E. and Joe Ghiold, The functional significance of the lunules in the sand dollar, *Mellita quinquiesperforata*, 561
Algae, 456, 478, 498, 500, 501
  and *Cassiopeia andromeda*, 394
Alkon, Daniel, Gastropod models of associative learning, 505
Allende, Gloria, and Nancy Dise. Food preferences and population dynamics of the amphipod *Talorchestia longicornis*, 455
Ambystoma blastomeres, 493
Amemiya, Shonan, see Takao Mori, 728
Amino acids, in *Glycera* at various salinities, 626
Anderson, P., *et al.*, Are membrane lipids calcium ionophores? A comparison, employing arsenazo III in liposomes, between known ionophores (A23187, ionomycin) and phospholipids, fatty acids, prostanoids, and retinoids, 479
Anderson, Robert S., Hemolysins and hemagglutinins in the coelomic fluid of a polychaete annelid, *Glycera dibranchiata*, 259
Anderson, Sara, see Francis W. Klotz, 496
Anglerfish, islet, 473, 476
Annual gonadal variation in sea urchins of the orders Echinothurioida and Echinoida, 728
Annual report of the Marine Biological Laboratory, 1
Anthomedusan, electrical activities in subtentacular region, 376
Application of a polyploid marker to clonal analysis in *Xenopus* eye, 455
Aquarium, maintenance of squid in, 319
Arbacia, 467, 468, 469, 473, 474, 476, 495
Are membrane lipids calcium ionophores? A comparison employing arsenazo III in liposomes, between known ionophores (A 23187, ionomycin) and phospholipids, fatty acids, prostanoids, and retinoids, 479
Are there membrane surface charges in the vicinity of the sodium pump? 487
Arias Negrete, Sergio, *et al.*, Solid phase radioimmune assay to detect antibodies in *Entamoeba histolytica*, 495
  see Francis W. Klotz, 496
Armstrong, Clay M., see Wm. F. Gilly, 483
  see J. Lopez-Barneo, 487
  see Rick Matteson, 488
  see R. P. Swenson, 493
Arnold, John M., and Lois D. Williams-Arnold, Development of the ciliature pattern on the embryo of the squid, *Loligo pealei*: A scanning electron microscope study, 102
Aronstein, Diane, see Keith Nelson, 162, 503
Arsenazo 122a, III
  reveals long-lasting intracellular calcium transient following the action potential in squid giant presynaptic terminal, 491
Ascidia ceratodes, blood plasma, 656
Ascidian, see *Styela montereyensis*, *Podoclavella moluccensis*, *Pyura stolonifera*, *Ascidia ceratodes*, *Symplegma reptans*

Ascidian blood chemistry, 656, 669
Aspects of the life history of *Carcinonemertes errans* (Nemertea: Carcinonemertidae), an egg predator of the crab *Cancer magister*, 247
Associative learning, in gastropods, 505
Asterias forbesi, see starfish
Atema, Jelle, see Charles Derby, 450
Axons, 471, 478, 481, 483, 484, 485, 487, 488, 491, 494

B

Bacteria, 456, 457, 459–463, 465
Bacterial epiphytes on *Zostera marina* surfaces, 461
Bacterial kidney disease of rainbow trout in sea water, 495
Ballinger, D., and T. Hunt, Further observations on the phosphorylation of ribosomal proteins after fertilization of *Arbacia punctulata* eggs, 473
Bang, Betsy G., and Frederik B. Bang, The urn cell complex of *Sipunculus nudus:* A model for study of mucus-stimulating substances, 571
and S. J. Cooperstein. Janus green B: A vital stain during the process of mucus secretion, 447
Bang, Frederik B., see Betsy G. Bang, 571
Inflammation in the sea star, *Asterias forbesi*, 495
Bannon, Gary A., and George Gordon Brown, Ultrastructural characteristics of the non-expanded and expanded extra-embryonic shell of the horseshoe crab, *Limulus polyphemus* L., 582
Barlow, Robert B., Jr., see Ehud Kaplan, 486
see Leonard Kass, 487
see Takehiko Saito, 490
Barnes, Edward, and Izja Lederhendler, Dark-adaptation effects on photobehavior of *Hermissenda crassicornis* (Gastropoda: nudibranchia), 479
Barokinesis, in crab *Callinectes sapidus* larvae, 402
Bartelt, Diana C., and William D. Cohen, Labile components of the dogfish erythrocyte cytoskeleton, 441
Bathynomus giganteus (sea roach), 478
Batra, Ranjan, see Ehud Kaplan, 486
Bauer, G. Eric, *et al.*, Subcellular localization and characterization of islet hormone-degrading enzymes in anglerfish islet tissue, 473
see Bryan D. Noe, 476
Baumgold, Jesse, and Paul Gallant, Release of cytoskeletal proteins into the perfusate of squid giant axons, 441
Behavioral basis of larval recruitment in the crab *Callinectes sapidus* Rathbun: A laboratory investigation of ontogenetic changes in geotaxis and barokinesis, 402
Bell, Wayne H., Microbial ecology of algal extra-cellular products: the specificity of alga-bacterial interactions, 456
Bennett, M. V. L., see J. H. Stern, 493
Berg, Carl J., see Gregory A. Tracey, 465
Better fluorescent probes for optical measurement of changes in membrane potential, 484
Bezanilla, F., see B. M. Salzberg, 491
Bigger, Charles H., Interspecific and intraspecific acrorhagial aggressive behavior among sea anemones: A recognition of self- and not-self, 117
Biggs, Wilton R., see Clifford J. Hawkins, 656
Bile pigments, 499, 501
Binding of dynein to isolated meiotic spindles of the surf clam, *Spisula solidissima*, 446
Biochemistry, abstracts, 473
Bioluminescence, see luminescence
Birefringence response of voltage-clamped internally perfused axons, 491
Blood chemistry of ascidians, 656, 669
Blue crab, see *Callinectes sapidus*
Bolsover, S. R., and J. E. Brown, Guanosine phosphates and the control of membrane potential in *Limulus* ventral photoreceptors, 480
Borgese, Joan M., see Thomas A. Borgese
Borgese, Thomas A., *et al.*, Thermostability of fish hemoglobins, 448
Borgeson, Will, see Keith Nelson, 162, 503
Bousquette, George D., The larval development of *Pinnixa longipes* (Lockington, 1877) (Brachyura: Pinnotheridae) reared in the laboratory, 592
Bowles, Francis, see Amy Friedlander, 458
see David W. Juers, 461
Boyer, Barbara, Mosaic development in the polyclad turbellarian *Hoploplana inquilina* and its evolutionary implications, 448
Boyle, M. B., and L. B. Cohen, Preliminary evidence for taste-aversion learning in the nudibranch mollusc *Aeolidia papillosa*, 480
Braithwaite, Lee F., see Criag M. Young, 428
Brandl, Helmut, *et al.*, Studies of methanogenic bacteria from intestinal tracts of marine fishes, 456
Brenchley, G. A., Distribution and migratory behavior of *Ilyanassa obsoleta* in Barnstable Harbor, 457
Brenowitz, M., see K. E. Van Holde, 478
Bretos, Marta, Age determinations in the keyhole limpet *Fissurella crassa*, 606
Brown, George Gordon, see Gary A. Bannon, 582
Brown, Jay C., see Lucio Cariello, 467
Brown, J. E., see S. R. Bolsover, 480

Brown, S. B., *et al.*, Phycocyanobilin synthesis from exogenous heme, 499
  see K. M. Smith, 501
  see R. F. Troxler, 502
Bruce, Victor G., see Judith E. Goodenough, 649
Budding, of *Cassiopeia andromeda*, effect of symbionts, 394
Bufo, neuroplasmic lattice, 471
Burger, Max M., see Werner Burkart, 449
Burgos, Mario H., *et al.*, Gossypol inhibits motility of *Arbacia* sperm, 467
  see Michael Coburn, 468
Burkart, Werner, and Max M. Burger, Substitution of calcium by polycations in sponge aggregation factor interaction, 449
Burrowing sponge, carbonic anhydrase implicated in mechanism of penetration, 135
Busa, W. B., *et al.*, Light-scattering studies of sheared and unsheared actin polymerization, 442

C

Cadmium resistance in bacteria from the Great Sippewissett Marsh, 463
Caffeine, effects on *Chlamydomonas reinhardii*, 649
Calcium, 449, 470, 480, 481, 485, 491, 494
  and potassium activities in the hemolymph of the squid, *Loligo pealei*, 492
Callinectes sapidus, behavioral basis of larval recruitment, 402
  mandibular gland, 760
Calmodulin from the axoplasm of the squid, 485
Campanularia flexuosa, 752
Cancer magister, predation on eggs by *Carcinonemertes errans*, 247
Cape Cod National Seashore, 466
Carbonate substrata, penetration of by burrowing sponge, 135
Carbon fixation, by *Prochloron* isolated from *Diplosoma virens*, 636
Carbonic anhydrase, in *Cliona celata* burrowing, 135
Carcinonemertes errans, crab-egg predator, life history, 247
Cariello, Lucio, *et al.*, Properties of isolated fertilization envelopes from *Arbacia punctulata*, 467
Carlisle, James C., and Jan Spitsbergen, Bacterial kidney diseases of rainbow trout in sea water, 495
Carter, R., see D. Wirth, 498
Cartesian diver, 692
Case, James F., Courting behavior in a synchronously flashing aggregative firefly, *Pteroptyx tener*, 613
  see John A. Warner, 231
Cassiopeia andromeda, budding and strobilation, 394
Cavanaugh, Colleen M., Symbiosis of chemoautotrophic bacteria and marine invertebrates, 456
Cell membrane, 471
Cell motility and cytoskeleton, abstracts, 441
Central projections of the lateral eyes of *Balanus nubilis* (Pacific giant barnacle) differ from those of the median eye, 483
Chambers, Randy, and Anthony Pires, The relationship between diet and growth rate of the grass shrimp *Palaemonetes pugio*, 458
Change in cyclic nucleotide content during germinal vesicle breakdown in *Spisula* oocytes, 466
Change in protein synthetic pattern and mRNA population during *Spisula* embryogenesis, 478
Chang, Ernest S., see Ashley I. Yudin, 760
Characterization of periodic order in the neuroplasmic lattice of *Bufo*, *Loligo*, and *Hermissenda* axons by a Fourier analytical technique in scanning transmission electron microscopy (STEM), 471
Charlton, Milton P., *et al.*, Presynaptic injection of calcium facilitates transmitter release in the squid giant synapse, 481
  see Stephen J Smith, 491
  see Robert S. Zucker, 494
Chemical communication in *Homarus*, 162
Chemical conversion of a chlorophyll *a* derivative to a bile pigment, 501
Chemistry of the blood of the ascidian *Podoclavella moluccensis*, 669
Chemoreceptors, on legs of *Homarus americannus*, 450
Chemotaxis, 443
Chih-Yi, Chang, see Mario H. Burgos, 467
Chironomus, 489
Chiton water balance and salinity stress, 364
Chlamydomonas reinhardii, effects of caffeine and theophylline on phototactic response of, 649
Chlorophyll *a*, 501, 502
Chromium, 462
  in ascidian blood, 669
Cilia, 444, 446
  pattern, on squid embryo, 102
Ciona, 446
Circadian rhythm of photoreceptor cells in the *Limulus* lateral eye: further studies, 490
Clams, freshwater, sodium regulation in, 325
Clark, W. H., Jr., see Ashley I. Yudin, 760
Cliona celata, carbonic anhydrase implicated in mechanism of burrowing, 135
Cloning genes in *Leishmania enriettii* in *E. coli*, 498
Coburn, Michael, *et al.*, Oxygen free radical generation by gossypol: a possible mechanism of antifertility action in sea urchin sperm, 468
  see Peter Sinsheimer, 469

Coelomic fluid, hemolysins and hemagglutinins in *Glycera*, 259
Coelomocytes, red, salinity tolerance and volume regulation in *Glycera*, 626
  ultrastructure, of *Dermasterias imbricata*, 295
Cohen, L. B., see M. B. Boyle, 480
  see A. Grinvald, 484
Cohen, William D., *et al.*, The marginal band system of the dogfish erythrocyte, 442
  see Diana C. Bartelt, 441
  see Iris Nemhauser, 444
Colorless proteins in phycobilisomes of rhodophycean and cyanobacterial species, 499
Color, of bodies of fishes in relation to environmental light, 450
Comparative study of the blood plasma of the ascidians *Pyura stolonifera* and *Ascidia ceratodes*, 656
Competition, in *Homarus*, 162
  in sea anemones, 117
  in *Teredo navalis*, 465
  resource partitioning in sympatric hermit crabs, 337
Contextual relationships in food webs involving meiofauna, 462
Control of drilling fluid discharge from petroleum development on Georges Bank, 463
Conway, Kevin, and R. Kevin Hunt, Healing of Xenopus eye fragments premarked by chimaeric pigment grafts, 453
  see R. Kevin Hunt, 453
  see Sarah A. Shoaf, 454
  see Robert Tompkins, 455
Cooperstein, S. J., see B. G. Bang, 447
Copeland, Eugene, *et al.*, Gas secretion in the swimbladder of shallow water fishes compared to a deep ocean fish (3000 meters), 449
Copepod, formation of siliceous teeth by *Acartia tonsa*, 349
  Labidocera aestiva, control of diapause, 311
Corbicula fluminea, sodium balance, 325
Corr, Michelle, and David Hughes, The predatory habits of two lycosid spiders in Great Sippewissett Marsh, 456
Corson, D. Wesley, and Alan Fein, A GTP binding component regulates discrete wave production in *Limulus* ventral photoreceptors: pharmacological evidence, 481
Costa, Charles J., *et al.*, The intracellular mechanism of salinity tolerance in polychaetes: Volume regulation by isolated *Glycera dibranchiata* red coelomocytes, 626
Coupling between horizontal cells in the carp retina examined by diffusion of Lucifer yellow, 486
Courting behavior in a synchronously flashing, aggregative firefly, *Pteroptyx tener*, 613
Crab, see *Callinectes sapidus*, *Cancer magister*, hermit crabs, *Panopeus herbstii*, *Pinnixa*, *Uca*
Crangon franciscorum, seasonal abundance and distribution, 177
Crayfish, see *Procamberus clarkii*
Crowding, effects in *Homarus*, 162
Crystalline axes of the test and spine of the sea urchin *Strongylocentrotus purpuratus*—a new method of analysis, 472
Ctenophores, 446
Cummings, Glenna D., see Jay E. Mittenthal, 714
Current, effects on ascidian *Styela montereyensis*, 428
Cyanobacteria, 498–500, 502, 636
Cytoskeleton, 441, 471
Czeto, A. R., *et al.*, An X-ray study of the retinal photoreceptor structure of squid, 482

D

Daggett, Richard, see Keith Nelson, 162
Dan, Katsuma, see Shinya Inoué, 443
Dark-adaptation effects on photobehavior of *Hermissenda crassicornis* (Gastropoda: nudibranchia), 479
David, John, see D. Wirth, 498
Davila, H. V., see B. M. Salzberg, 491
Dawson, Benjamin G., *et al.*, Field experiments on electrically evoked feeding response in the dogfish shark, *Mustelus canis*, 482
Dendritic spine necks, 470
Denitrification potentials in a successional sequence of northern hardwood forest stands, 464
Density-dependent growth inhibition in lobsters, *Homarus* (Decapoda, Nephropidae), 162, 503
Derby, Charles, and Jelle Atema, L-Glutamate-specialist chemoreceptors on the legs of the lobster *Homarus americanus*, 450
DeRiemer, Susan A., and Eduardo R. Macagno, Positional correlation of synaptic boutons in pairs of mechanosensory cells in the leech, 482
Dermasterias imbricata, ultrastructure of coelomocytes, 295
DeSantis, Rosaria, see David Stopak, 446
Developmental analysis of an unusual homoeotic mutation, proboscipedia, in *Drosophila melanogaster*, 454
Development of the ciliature pattern on the embryo of the squid, *Loligo pealei:* A scanning electron microscope study, 102
De Weer, Paul, see George R. Kracke, 487
Diapause, photoperiod control in *Labidocera aestiva*, 311
Dietz, Thomas H., see Susan McCorkle, 325

DIENER, RICHARD A., see Ashley I. Yudin, 760
DILLAMAN, RICHARD, see Kayo Okazaki, 472
Dinurus, 737
Diplosoma virens, photosynthesis by *Prochloron* isolated from, 636
DISE, NANCY, see Gloria Allende, 455
Distribution and migratory behavior of *Ilyanassa obsoleta* in Barnstable Harbor, 457
Dogfish, 441, 442, 451, 452, 472, 482
Dormancy, of copepod *Labidocera aestiva*, 311
Doryteuthis bleekeri, maintenance in aquaria, 319
Drosophila melanogaster, proboscipedia, in, 454
Drug delivery to dogfish lens, 472
DUBOIS, ARTHUR B., AND CHRISTOPHER S. OGILVY, Thrust and drag of bluefish (*Pomatomus saltatrix*) at different buoyancies, speeds, and swimming angles, 450
  see Christopher S. Ogilvy, 451
DUNHAM, P., see P. Anderson, 479

E

Ecdysone, and terrestrial isopod crustacea, 337
Echinoderms, see starfish
Echinoida, sea urchins, reproductive cycle, 728
Echinothurioida, sea urchins, reproductive cycle, 728
ECKMAN, STEVE, see R. Kevin Hunt, 453
Ecology, abstracts, 455
Effectiveness of National Park Service policies in protecting barrier island ecosystems within the Cape Cod National Seashore, 466
Effect, of chromium on microbial activity in salt marsh sediments, 462
  of cytochalasin B and phalloidin on F-actin and G-actin, 449
  of grazing by *Uca pugnax* on the microbial population in salt marsh sediment, 465
  of sewage fertilization on benthic macroinvertebrates in salt marsh creeks, 465
  of symbiotic zooxanthellae and temperature on budding and strobilation in *Cassiopeia andromeda* (Eschscholz), 394
  of temperature acclimation on nitrogen metabolism in two littorinid snails, 447
Effects, of caffeine and theophylline on the phototactic thythm of *Chlamydomonas reinhardii*, 649
  of closed-culture competitive interactions on growth of *Teredo Navalis* larvae, 465
  of magnetic fields on regeneration in fiddler crabs, 681
  of media with low silicic acid concentrations on tooth formation in *Acartia tonsa Dana* (Copepoda, calanoida), 349
  of temperature and salinity on the osmotic composition of the southern oyster drill, *Thais haemastoma*, 148
Electrical, activities in the subtentacular region of the anthomedusan *Spirocodon saltatrix* (Tilesius), 376
  membrane properties of single and two-cell preparations from *Chironomus* salivary gland, 489
Electron spin resonance studies of protein-methylglyoxal complexes, 474
Electrophoretic variation in sympatric mud crabs from North Inlet, South Carolina, 418
Electrophysiology, of *Spiricodon saltatrix*, 376
  Abstracts, of MBL General Meetings, 441 ff.
Elimination of synapses from identified lobster motor neurons during development, 492
ELLISON, REBECCA P., ADP ribosylation in nuclei isolated from different embryonic stages of *Arbacia*, 474
Embryogenesis, of squid, 259
Embryonic development, of *Limulus*, 582
Entamoeba histolytica, 495, 496
Enzyme activity in early embryos, 475
EPPI, RENÉ E., see Benjamin G. Dawson, 482
Erythrocytes, 441
Europanoepus depressus, electrophoretic variation in, 418
Evaluation of low light level intensifier vidicon detectors for microscopy, 473
External K^+ and Rb^+ retarded closing of potassium channels, 493
Extra-embryonic shell of *Limulus*, 582
Eye, see photoreceptors, lens

F

FARMANFARMAIAN, A., *et al.*, Mechanism of heavy metal inhibition of amino acid transport in the intestine of marine fishes, 458
Feasibility of seaweed aquaculture in the Great Sippewissett Salt Marsh, 459
Feeding, and induction of bioluminescence in *Porychthys notatus*, 231
  of *Neomysis mercedis* (Holmes), 193
  of *Styela montereyensis*, 428
FEIN, ALAN, see D. Wesley Corson, 481
Fertilization, abstracts on, 466 ff., 473, 474, 478
Fiddler crabs, see *Uca*
Field experiments on electrically evoked feeding response in the dogfish shark, *Mustelus canis*, 482
Firefly, flashing and courting, 613
FISHER, CHARLES R., JR., AND ROBERT K. TRENCH, *In vitro* carbon fixtion by *Prochloron* sp. isolated from *Diplosoma virens*, 636
Fishes, 449–451, 457, 458, 485, 486, 495
  see also dogfish, toadfish, *Porychthys notatus*
Fissurella crassa, shell growth rings and age determination, 606

Fohlmeister, Jurgen F., see William J. Adelman, Jr., 478
Food choice and palatability in a salt marsh detritivore, *Melampus bidentatus*, 464
Food preferences and population dynamics of the amphipod *Talorchestia longicornis*, 455
Formation and early differentiation of sea urchin gonads, 280
Fourier analytical technique, characterizing order in neuroplasmic lattice, 471
Freeze-fracturing, 470, 471
French, C., see D. Wirth, 498
French, Kathleen A., and Ann E. Stuart, The central projections of the lateral eyes of *Balanus nubilis* (Pacific giant barnacle) differ from those of the median eye, 483
Friedlander, Amy, *et al.*, Mapping vegetation and topography of Great Sippewissett Salt Marsh, Mass., 458
Frog, see *Xenopus*
Fujita, Rodney M., The feasibility of seaweed aquaculture in the Great Sippewissett Salt Marsh, 459
Functional significance of the lunules in the sand dollar, *Mellita quinquiesperforata*, 561
Fundulus, 493
Further improvement upon maintenance of adult squid (*Doryteuthis bleekeri*) in a small circular and closed-system aquarium tank, 319
Further observations on the phosphorylation of ribosomal proteins after fertilization of *Arbacia punctulata* eggs, 473
Futrelle, R. P., The theory of chemotaxis and the ability of a cell to sense its position and orientation within a tissue, 443

G

Gale, Judith, see Amy Friedlander, 458
Gallant, Paul, see Jesse Baumgold, 441
Gamma radiation and hydranth longevity in *Campanularia flexuosa:* Age-dependency of dose-response function, 752
Ganglion ablation and gonad development in ascidian *Symplegma reptans*, 219
Gap junctions: quantitative comparison of reduction in conductance by H and by Ca ions in an internally perfused preparation, 493
Gardner, Karen, see Judith Megaw, 472
Gascoyne, Peter, *et al.*, Electron spin resonance studies of protein-methylglyoxal complexes, 474
  see Ronald Pethig, 477
Gas secretion in the swimbladder of shallow water fishes compared to a deep ocean fish (3000 meters), 449
Gastropod models of associative learning, 505
Genetic differences, in sympatric mud crabs, 418
Genetics, abstracts, 453
Geotaxis, in crab *Callinectes sapidus* larvae, 402
Germinal vesicle breakdown, 466, 468
Ghiold, Joe, see David E. Alexander, 561
Gilbert, Daniel L., see Roderic E. Steele, 492
Gilly, Wm. F., and Clay M. Armstrong, Interaction of external transition metal ions and the mobile gating charges of Na and K channels in squid axon, 483
Gilson, Michael K., and Ad. J. Kalmijn, Statistical mechanics of geomagnetic orientation in sediment bacteria, 459
Gitler, Carlos, see Eileen Lynch, 496
  see Willy F. Piessens, 497
  see D. Wirth, 498
L-Glutamate-specialist chemoreceptors on the legs of the lobster *Homarus americanus*, 450
Glycera, humoral immunity, 259
  salinity tolerance, 626
  venom and axons, 485
Gonad development and ganglion ablation in ascidian *Symplegma reptans*, 219
Gonads, annual cycle in Echinoida and Echinothurioida, 728
  sea urchin, formation and early differentiation, 280
Goodenough, Judith E., and Victor G. Bruce, The effects of caffeine and theophylline on the phototactic rhythm of *Chlamydomonas reinhardii*, 649
Goodman, E., see P. Anderson, 479
Gossypol, 467, 468
Gould, Robert M., Phospholipid synthesis in axoplasm from the squid giant fiber, 484
Govind, C. K., see Philip J. Stephens, 492
Graham, Stephen, *et al.*, Larval settlement on microbial films: A model system, 460
Greenberg, E. P., see Frederick H. Weber, 465
  see Helmut Brandl, 457
Grinvald, A., *et al.*, Better fluorescent probes for optical measurement of changes in membrane potential, 136
Growth inhibition in lobsters, 162, 503
Growth rings, in *Fissurella crassa* shell, 606
GTP binding component regulates discrete wave production in *Limulus* ventral photoreceptors: pharmacological evidence, 481
Guanosine phosphates and the control of membrane potential in *Limulus* ventral photoreceptors, 480
Guillard, Robert, R. L., see Charles B. Miller, 349
Gupta, R., see A. Grinvald, 484

H

Habitat, *Styela montereyensis*, 428
  see also ecology
Hadlock, Robin P., Alarm response of the intertidal snail *Littorina littorea* (L.) to

predation by the crab *Carcinas maenas* (L.), 269
HAEDRICH, RICHARD, see Eugene Copeland, 449
HAIMO, LEAH T., see Bruce R. Telzer, 446
HANNA, ROBERT B., see Bruce L. Kagan, 485
HARRINGTON, JOHN P., see Thomas A. Borgese, 448
HARRIS, A. L., see J. H. Stern, 493
HARRIS, ANDY, see Eileen Lynch, 496
HARRIS, KRISTEN, Relationships between dendrite and spine neck diameters in freeze-fractured rat hippocampal formation, 470
HASCHEMEYER, AUDREY E. V., see Rita W. Mathews, 475
HATCH, WALTER I., The implication of carbonic anhydrase in the physiological mechanism of penetration of carbonate substrata by the marine burrowing sponge *Cliona celata* (Demospongiae), 135
HAWKINS, CLIFFORD J., *et al.*, Chemistry of the blood of the ascidian *Podoclavella moluccensis*, 669
HAWKINS, CLIFFORD J., *et al.*, Comparative study of the blood plasma of the ascidians *Pyura stolonifera*, and *Ascidia ceratodes*, 656
HEAD, JAMES F., AND BENJAMIN KAMINER, Calmodulin from the axoplasm of the squid, 485
Healing of *Xenopus* eye fragments premarked by chimaeric pigment grafts, 453
HEDGECOCK, DENNIS, see Keith Nelson, 162
Hemagglutinins in the coelomic fluid of *Glycera dibranchiata*, 259
Hemocyanin, of sea roach, 478
Hemoglobins, fish thermostability, 448
Hemolysins and hemmagglutinins in the coelomic fluid of a polychaete annelid, *Glycera dibranchiata*, 259
Hermissenda, 471, 479
  associative learning in, 505
Hermit crabs, specificity of association with *Hydractinia*, 337
HEYER, GAIL W., see Benjamin G. Dawson, 482
HILDESHEIM, R., see A. Grinvald, 484
HILDRETH, JANE E., AND WILLIAM B. STICKLE, The effects of temperature and salinity on the osmotic composition of the southern oyster drill, *Thais haemastoma*, 148
HINEGARDNER, RALPH T., see Margaret S. Houk, 280
HINES, MICHAEL, see John Moore, 488
Histochemical identification of N-acetyl-β-glucosaminidase activity in early embryos of *Lytechinus pictus*, 475
HODGE, ALAN J., AND WILLIAM J. ADELMAN, JR., Characterization of periodic order in the neuroplasmic lattice of *Bufo*, *Loligo*, and *Hermissenda* axons by a Fourier analytical technique in scanning transmission electron microscopy (STEM), 471
Homarus, see lobster
HOMMEL, M., see D. Wirth, 498
Hoploplana inquilina, mosaic development, 448
HORGAN, ERICH F., AND MARGARET S. RACE, The relationship between organic and nitrogen content of marsh sediments and feeding rates in *Uca pugnax*, 460
Hormone-degrading enzymes in anglerfish islet tissues, 473
Horseshoe crab, see *Limulus*
HORWITZ, I. S., see G. Salama, 449
HOUK, MARGARET S., AND RALPH T. HINEGARDNER, The formation and early differentiation of sea urchin gonads, 280
HUFNAGEL, LINDA, Oriented particle assemblies in the plasma membrane of *Tetrahymena:* their deployment relative to cell surface topography, cellular morphogenesis and sensitivity to stimuli, 471
HUGHES, DAVID, AND MICHAEL USEM, Preferred food sources and the limitation of local distributions of the isopod crustacean *Philoscia vittata*, 460
  see Michelle Corr, 456
HUNT, R. KEVIN, see Kevin Conway, 453
  et al., Pigmentation mosaicism in the choroid of the eye after embryonic grafting, 453
  et al., Retinotectal patterns and patterns of genetic mosaicism of ploidy chimerae of *Xenopus* eye, 454
  see Sarah A. Shoaf, 454
  see Robert Tompkins, 455
HUNT, T., see D. Ballinger, 473
Hydractinia, symbiosis with hermit crabs, 337
Hydranth longevity and gamma radiation in *Campanularia flexuosa*, 752
Hydrozoan, see *Hydractinia*, *Spirocodon saltatrix*

I

Ilyanassa obsoleta, distribution and migration, 457
Immune response, of *Dermasterias imbricata*, 295
Immunology, abstracts, 495
  annelid, 259
Implication of carbonic anhydrase in the physiological mechanism of penetration of carbonate substrata by the marine burrowing sponge *Cliona celata* (Demospongiae), 135
Inflammation in the sea star, *Asterias forbesi*, 495
Inhibition, of islet prohormone to hormone conversion by incorporation or arginine and lysine analogs, 476
  of L-leucine transport in toadfish liver by various natural amino acids, 477

INOUÉ, SHINYA, AND KATSUMA DAN, Mitotic spindle behavior in unequal cleavage of *Spisula solidissima*, 466
INOUYE, H., *et al.*, An X-ray study of fish nerves, 485
Inside-out voltage clamp in the squid giant axon, 487
Interaction, of external transition metal ions and the mobile gating charges of Na and K channels in squid axon, 483
  of phalloidin with G- and F-actin, 445
Interactions between two species of littorines—*Littorina littorea* and *L. Saxatilis*—along New England shores, 463
Interspecific and intraspecific acrorhagial aggressive behavior among sea anemones: A recognition of self- and not-self, 117
Interstitial fluid pressures of smooth dogfish (*Mustelus canis*) and bluefish (*Pomatomus saltatrix*) tilted in air, 451
Intracellular mechanism of salinity tolerance in polychaetes: Volume regulation by isolated *Glycera dibranchiata* red coelamocytes, 626
In vitro carbon fixation by *Prochloron* sp. isolated from *Diplosoma virens*, 636
Ion and water balance of the hypo- and hyper-osmotically stressed chiton *Mopalia muscosa*, 364
  permeable channel produced by venom of the fanged bloodworm *Glycera dibranchia*, 485
  regulation in freshwater clams, 325
  regulation in *Thais haemastoma*, 148
Iron, in ascidian blood plasma, 656
Islet tissue, 473, 476
Isopods, terrestrial, coordination of moulting and reproduction, 206
  see *Bathynomus giganteus*
IWASA, K., see I. Tasaki, 494

J

JAEGER, RICHARD, see William D. Cohen, 442
Janus green B: A vital stain during the process of mucus secretion, 447
  and urn cell mucus, 571
JOHNSON, ERIC, see Keith Nelson, 162, 503
JUERS, DAVID W., *et al.*, Tidal water exchanges between Great Sippewissett Salt Marsh and Buzzards Bay, 461

K

KAGAN, BRUCE L., *et al.*, An ion-permeable channel produced by venom of the fanged bloodworm *Glycera dibranchia*, 485
KAHLER, STEPHEN, see G. Eric Bauer, 473
  see Bryan D. Noe, 476
KALMIJN, AD. J., see Michael K. Gilson, 459
  see Benjamin G. Dawson, 482
KAMINER, BENJAMIN, see James F. Head, 485
KANEKO, AKIMICHI, AND ANN E. STUART, Coupling between horizontal cells in the carp retina examined by diffusion of Lucifer yellow, 486
KANESHIRO, EDNA S., AND RICHARD D. KARP, The ultrastructure of coelomocytes of the sea star *Dermasterias imbricata*, 295
KAPLAN, EHUD, *et al.*, Recording from the *Limulus* ventral eye *in situ:* is there a circadian rhythm? 486
  see Takehiko Saito, 490
KARP, RICHARD D., see Edna S. Kaneshiro, 295
KASS, LEONARD, AND ROBERT B. BARLOW, JR., Octopamine increases the ERG of the *Limulus* lateral eye, 487
KAUFMAN, THOMAS C., see Anna W. Seitz, 454
KELLY, P., see S. D. Sulkin, 402
KEM, WILLIAM R., AND JAMES D. SCOTT, Partial purification and characterization of a cytotoxic protein from squid (*Loligo pealei*) posterior salivary glands, 475
KIRCHMAN, DAVID, see Stephen Graham, 460
KIRCHMAN, D. L., *et al.*, Bacterial epiphytes on *Zostera marina* surfaces, 461
KLOTZ, FRANCIS W., *et al.*, Schistosomiasis immune evasion, 496
KOECH, DAVID, see Francis W. Klotz, 496
KOIDE, SAMUEL S., see Oyewole Adeyomo, 466
  see Akira Momii, 468
  see Aloys G. Tumboh-Oeri, 470
KOPACHE, MARK E., Feeding of *Neomysis mercedis* (Holmes), 193
KRACKE, GEORGE R., AND PAUL DE WEER, Are there membrane surface charges in the vicinity of the sodium pump? 487
KURSAR, T. A., see R. S. Alberte, 498
  et al., Relationships between photosystem II and phycobilisomes in red algae and cyanobacteria, 500
  see Scott Schatz, 501

L

Labeling of microfilariae of *Brugia malayi*, 497
Labile components of the dogfish erythrocyte cytoskeleton, 441
Lack of respiratory response to temperature acclimation in two littorinid snails, 452
LANDOWNE, DAVID, see Virginia Scruggs, 491
Larval behavior, *Styela montereyensis*, 428
Larval development, geotaxis and barokinesis, and recruitment in crab *Callinectes sapidus*, 402
  of *Pinnixa longipes* (Lockington, 1877) (Brachyura: Pinnotheridae), reared in the laboratory, 592
Larval respiration, and metabolism, of *Macrobrachium olfersii*, 692
Larval settlement, on microbial films: A model system, 460
Lasiotocus minutus, 497

Learning, 480
  associative, in gastropods, 505
LEDERHENDLER, IZJA, see Edward Barnes, 479
LEE, JOHN J., AND MONICA J. LEE, Contextual relationships in food webs involving meiofauna, 462
LEE, MONICA J., see John J. Lee, 462
LEE, PAUL H., AND JUDITH S. WEIS, Effects of magnetic fields on regeneration in fiddler crabs, 681
Leech, mechanosensory cells, 483
LEIGHTY, BRUCE, Effect of chromium on microbial activity in salt marsh sediments, 482
Leishmania enrietii, 497, 498
  Lens (dogfish), 452, 472
LERMAN, SIDNEY, see Judith Megaw, 472
LEVINE, JOSEPH S., AND EDWARD F. MACNICHOL, JR., Relationship of body colors to environmental light conditions in "poster colored" fishes, 450
Life history, of *Carcinonemertes errans*, 247
  of copepod *Labidocera aestiva*, 311
  of *Lasiotocus minutus*, 497
  of shrimp *Crangon franciscorum* and *Palaemon macrodactylus*, 177
  of *Tubulovesicula pinguis*, 737
Light-scattering studies of sheared and unsheared actin polymerization, 442
Limpet, *Fissurella crassa*, age determination, 606
Limulus, 480, 481, 486, 487, 490, 582
Liposome intraocular drug delivery, 472
LIPSCHULTZ, FREDRIC, see Annette Spies, 465
Littorina littorea and *saxatilis*, interactions, 463
Lobster, 450, 492
  (*Homarus*), density-dependent growth inhibition, 162, 503
Loligo, see squid
LÓPEZ-BARNEO, J., *et al.*, Inside-out voltage clamp in the squid giant axon, 487
Luminescence, diet and induction of in *Porychthys notatus*, 364
  firefly courting, 613
Lunules, function in *Mellita*, 561
LYERLA, TIMOTHY, see Katherine Turner, 418
LYNCH, EILEEN, *et al.*, A natural protozoan-derived ionophore: a possible mechanism of cytotoxicity by *Entamoeba histolytica*, 496
Lytechinus pictus, enzyme activity in early embryo, 475
  formation and early differentiation of gonads, 280
LYTLE, CHARLES F., see Neil A. Mercando, 337

M

MACAGNO, EDUARDO R., see Susan A. DeRiemer, 483
MACNICHOL, EDWARD F., JR., see Joseph S. Levine, 450
Macrobrachium olfersii (shrimp), larval respiration, 692
Magelona, 451
Magnetic, orientation in bacteria, 459
  fields, and fiddler-crab regeneration, 681
Mandibular gland of the blue crab, *Callinectes sapidus*, 760
MANSOUR, RANDA A., Interactions between the two special of littorines—*Littorina littorea* and *L. saxatilis*—along New England shores, 463
Mapping vegetation and topography of Great Sippewissett Salt Marsh, Mass., 458
MARCUS, NANCY H., Photoperiodic control of diapause in the marine calanoid copepod *Labidocera aestiva*, 311
Marine Biological Laboratory, annual report, 1
MATHEWS, RITA W., AND AUDREY W. V. HASCHEMEYER, Studies of protein synthesis in isolated toadfish hepatocytes, 475
Mating behavior in *Pteroptyx tener* (firefly), 613
MATSUMOTO, GEN, AND JUNICHI SHIMADA, Further improvements upon maintenance of adult squid (*Doryteuthis bleekeri*) in a small circular and closed-system aquarium tank, 319
MATTESON, R. D., see J. López-Barneo, 487
  and Clay M. Armstrong, Temperature effects on peak and steady state sodium currents in squid giant axons, 488
MAUZERALL, D., see L. Mazzella, 500
  see T. A. Kursar, 500
MAZZELLA, L., *et al.*, Photosynthetic light adaptation features of *Zostera marina* L. (eelgrass), 500
  see D. L. Kirchman, 461
MCCARTHY, MICHAEL P., Effects of cytochalasin B and phalloidin on F-actin and G-actin, 449
MCCORKLE, SUSAN, AND THOMAS H. DIETZ, Sodium transport in the freshwater Asiatic clam, *Corbicula fluminea*, 325
MCLAUGHLIN, JANE, see Peter Gascoyne, 474
  see Ronald Pethig, 477
MCMAHON, ROBERT F., *et al.*, Respiration in the polychaete worm *Magelona:* responses to temperature, hypoxia and tentacle ablation, 451
  see David W. Aldridge, 447
  see W. D. Russell-Hunter, 452
MCMAHON-PRATT, DIANNE, see Sergio Arias-Negrete, 495
  and students of parasitology course, Monoclonal antibodies to *Leishmania enriettii* and tubulin, 497
  see D. Wirth, 498
MCNAMARA, JOHN C., *et al.*, Respiratory metabolism of *Macrobrachium olfersii* (Wiegmann) zoeae during the moulting cycle from eclosion to first ecdysis, 692
Mechanics and energetics of contraction in striated muscle of the sea scallop, *Placopecton magellanicus*, 489

Mechanism, of heavy metal inhibition of amino acid transport in intestine of marine fishes, 458
  of intra-plate ciliary synchrony in ctenophores, 446
  of nicotinamide action in germinal vesicle breakdown in oocytes of *Spisula solidissima*, 468
Mechanisms of coordination between moulting and reproduction in terrestrial isopod Crustacea, 206
Megaw, Judith, *et al.*, Liposome intraocular drug delivery, 472
Meiofauna, food webs, 462
Melampus bidentatus, food choice, 464
Melillo, J. M., see J. P. Schimel, 464
Mellita quinquiesperforata, 561
Mercando, Neil A., and Charles F. Lytle, Specificity in the association between *Hydractinia echinata* and sympatric species of hermit crabs, 337
Merefield, Pauline M., see Clifford J. Hawkins, 656
Metals, in ascidian blood, 656, 669
Microanatomy and microtechniques, abstracts, 470
Microbial ecology of algal extracellular products: the specificity of alga-bacterial interactions, 456
Midshipman fish, see *Porichthys notatus*, 231
Miller, Charles B., David M. Nelson, Robert R. L. Guillard, and Bonnie L. Woodward, Effects of media with low silicic acid concentrations on tooth formation in *Acartia tonsa* Dana (Copepoda, Calanoida), 349
Miller, L., see D. Wirth, 498
Mischke, D., see D. Wirth, 498
Mitchell, R., see D. L. Kirchman, 461
Mitchell, Ralph, see Stephen Graham, 460
Mitochondrial movements in early development of *Ciona*, 446
Mittenthal, Jay E., *et al.*, Morphology of the closer muscles in normal and homoeotic legs of crayfish, 714
Mittenthal, Jay E., On the form and size of crayfish legs regenerated after grafting, 700
Molecular biology, abstracts, 473
Mollura, F., Cadmium resistance in bacteria from the Great Sippewissett Marsh, 463
Mollura, Francesca, see Helmut Brandl, 457
Momii, Akira, *et al.*, Mechanism of nicotinamide action on germinal vesicle breakdown in oocytes of *Spisula solidissima*, 468
Momii, F., see Akira Momii, 468
Monk, Peter B., see Anna W. Seitz, 454
Monoclonal antibodies to *Leishmania enriettii* and tubulin, 497
Moore, John, *et al.*, Oscillation-free compensation for the series resistance in voltage clamped squid axons, 488
Mooseker, M. S., see W. B. Busa, 441
  see Francine R. Smith, 445
Mopalia muscosa, salinity stress, 364
Moran, Michael N., see G. Eric Bauer, 473
  see Bryan D. Noe, 476
Moran, William M., and Richard E. Tullis, Ion and water balance of the hypo- and hyperosmotically stressed chiton *Mopalia muscosa*, 364
Moreira, Gloria S., see John C. McNamara, 692
Moreira, Plinio, see John C. McNamara, 692
Morgan, Kathleen, Histochemical identification of N-acetyl-β-glucosaminidase activity in early embryos of *Lytechinus pictus*, 475
Mori, Takao, *et al.*, Annual gonadal variation in sea urchins of the orders Echinothurioida and Echinoida, 728
Morphology, life history, and systematic relations of *Tubulovesicula pinguis* (Linton, 1940) Manter, 1947 (Trematoda: Hemiuridae), 737
  of crayfish legs, 700, 714
  of mandibular gland of *Callinectes sapidus*, 760
  of *Styela montereyensis*, 428
  of the closer muscles in normal and homoeotic legs of crayfish, 714
Mosaic development in the polyclad turbellarian *Hoploplana inquilina* and its evolutionary implications, 448
Mosaicism of pigmentation in frog eye, 453, 454
Motility, and squid embryo, 102
  Abstracts, 441
Moulting, coordination with reproduction in terrestrial isopods, 206
  cycle and metabolism of *Macrobrachium olfersii* larvae, 692
  influence of mandibular gland of blue crab, 760
Mucus secretion, and Janus green, 447
  in urn cell complex of *Sipunculus nudus*, 571
Mud crabs, electrophoretic variation in, 418
Mullin, Elizabeth, Control of drilling fluid discharge from petroleum development on Georges Bank, 463
Murray, Andrew, and Ronald Sosnowski, The status of mRNA in eggs and early embryos, 476
Muscle, in crayfish legs, 700, 714
Mutation, in *Drosophila*, 454

N

Nagel, Ronald L., see Thomas A. Borgese, 448

Natural protozoan-derived ionophore: a possible mechanism of cytotoxicity by *Entamoeba histolytica*, 496
Nelson, David M., see Charles B. Miller, 349
Nelson, Keith, Dennis Hedgecock, Will Borgeson, Eric Johnson, Richard Daggett, and Diane Aronstein, Density-dependent growth inhibition in lobsters, *Homarus* (Decapoda: Nephropidae), 162, 503
Nelson, Leonard, see Mario H. Burgos, 467
  Receptors, drug interactions, and calcium in regulation of *Arbacia sperm* cell function, 469
Nemhauser, Iris, and William D. Cohen, *In vivo* disassembly/reassembly of the marginal band in an invertebrate erythrocyte, 449
Neomysis mercedis feeding, 193
Neurobiology, abstracts, 478 ff.
  of *Hermissenda*, 505
Neuroplasmic lattice, 471
Neurosecretory cells and neural complex related to gonad development in ascidian *Symplegma* reptans, 219
Neustadt, Jeffrey B., see G. Eric Bauer, 473
  see Bryan D. Noe, 476
Nicotinamide action in germinal vesicle breakdown, 468
Ninhydrin positive substances, and *Thais haemastoma*, 148
Nitrogen metabolism, in littorinid snails, 447
Noe, Bryan D., see G. Eric Bauer, 473
  et al., Inhibition of islet prohormone to hormone conversion by incorporation of arginine and lysine analogs, 476

O

Obaid, Ana Lia, and Birgit Rose, Electrical membrane properties of single and two-cell preparations from *Chironomus* salivary gland, 489
Observations on the isolated mitotic apparatus ghost, 445
Octopamine increases the ERG of the *Limulus* lateral eye, 487
Ogilvy, Christopher S., and Arthur B. DuBois, Interstitial fluid pressures of smooth dogfish (*Mustelus canis*) and bluefish (*Pomatomus saltatrix*) tilted in air, 451
  see Arthur B. DuBois, 450
Ohtsu, Kohzoh, Electrical activities in the subtentacular region of the anthomedusan *Spirocodon saltatrix* (Tilesius), 376
Okazaki, Kayo, and Richard Dillaman, Crystalline axes of the test and spine of the sea urchin *Strongylocentrotus purpuratus*—A new method of analysis, 472
Olsen, Mary C., see Jay E. Mittenthal, 714
On the form and size of crayfish legs regenerated after grafting, 700
Opsanus tau, see toadfish
Optical determination of the resistance in series with the axolemma of *Loligo pealei*, 491
Orientation and current-induced flow in the stalked ascidian *Styela montereyensis*, 428
Oriented particle assemblies in the plasma membrane of *Tetrahymena:* their deployment relative to cell surface topography, cellular morphogenesis, and sensitivity to stimuli, 471
Orthogonal polarization sensitivities of squid photoreceptors: implication for a retinal design, 490
Oscillation-free compensation for the series resistance in voltage-clamped squid axons, 488
Osmoregulation, and freshwater clam, 325
  and chiton, 364
  in *Thais haemastoma*, 148
  in *Glycera*, 626
Oyster drill, *Thais haemastoma*, 148
Oxygen free radical generation by gossypol: a possible mechanism of antifertility action in sea urchin sperm, 468

P

Paguridae, symbiosis with *Hydractinia*, and shell selection, 337
Palaemonetes pugio, relationship between diet and growth, 458
Palaemon macrodactylus, seasonal abundance and distribution, 177
Panopeus herbstii, electrophoretic variation in, 418
Parasites, *Lasiotocus minutus*, 497
  Tubulovesicula pinguis, 737
Parasitology, abstracts, 495
Pardue, M., see D. Wirth, 498
Parry, David L., see Clifford J. Hawkins, 656, 669
Partial purification and characterization of a cytotoxic protein from squid (*Loligo pealei*) posterior salivary glands, 479
Paterek, J. R., see Helmut Brandl, 457
Paxhia, Teresa, see Seymour Zigman, 452
Persell, Roger, and Audrey E. V. Haschemeyer, Inhibition of L-leucine transport in toadfish liver by various natural amino acids, 477
Peterson, Bruce, see Amy Friedlander, 458
  see David W. Juera, 461
Pethig, Ronald, *et al.*, Influence of water structure on proton diffusion in proteins, 477
Petroleum drilling effects, 463
Phagocytosis, of *Dermasterias imbricata*, 295
Phalloidin, 444, 445
Philoscia vittata, 460
Phospholipid synthesis in axoplasm from the squid giant fiber, 484

Photoperiod, and terrestrial isopod crustacea, 206
Photoperiodic control of diapause in the marine calanoid copepod *Labidocera aestiva*, 311
Photoreceptors, 480, 481, 483, 486, 487, 490
  in *Hermissenda*, 505
Photosynthesis, abstracts, 498 ff.
  by *Prochloron* isolated from *Diplosoma virens*, 636
Photosynthetic activity of fungal infected and noninfected *Laminaria saccharina* Lam., 501
Photosynthetic light adaptation features of *Zostera marina* L. (eelgrass), 500
Phototactic response, effects of caffeine and theophylline on *Chlamydomonas reinhardii*, 649
Phycocyanobilin synthesis from exogenous heme, 499
Physiology, abstracts, 447
Pierce, Craig, see Clifford J. Hawkins, 669
Pierce, Sidney K., see Charles J. Costa, 626
Piessens, Willy F., Labeling of microfilariae of *Brugia malayi*, 497
  see Sergio Arias-Negrete, 495
Pigmentation mosaicism in the choroid of the eye after embryonic grafting, 453
Pinnixa longipes, larval development, 582
Pires, Anthony, see Randy Chambers, 458
Placopecton magellanicus (sea scallop), 489
Plant pigments and photosynthesis, abstracts, 498
Plasma, of *Pyura stolonifera* and *Ascidia ceratodes* blood, compared, 656
Podoclavella moluccensis, 669
Polidore, Thomas, see A. Farmanfarmaian, 458
Pollard, Harvey B., see Bruce L. Kagan, 140
Polychaete, see *Glycera*
Pomatomus saltatrix, see bluefish
Positional correlation of synaptic boutons in pairs of mechanosensory cells in the leech, 483
Potassium channels, 483, 485, 492, 493
Predation, by *Carcinonemertes errans* on *Cancer magister*, 247
  see also food, feeding
Predatory habits of two lycosid spiders in Great Sippewissett Marsh, 456
Preferred food sources and the limitation of local distributions of the isopod crustacean *Philoscia vittata*, 460
Preliminary, characterization of the hemocyanin of the giant sea roach, *Bathynomus giganteus*, 478
  evidence for taste-aversion learning in the nudibranch mollusc *Aeolidia papillosa*, 480
Presynaptic, calcium currents and facilitated transmitter release in the giant synapse of *Loligo pealei*, 494
  injection of calcium facilitates transmitter release in the squid giant synapse, 481
Primordial germ cells, of sea urchins, 280
Procambarus clarkii, see crayfish
Prochloron, photosynthetic capacity isolated from *Diplosoma virens*, 636
Proline, in *Glycera*, 626
Properties of isolated fertilization envelopes from *Arbacia punctulata*, 467
Protein, complexes, 474
  cytotoxic, 475
  plant, 498, 500
  proton diffusion, 477
  synthesis, 475, 478
  calmodulin, 485
Pteroptyx tener, (firefly) courting and flashing, 582
Purification of parasite mRNA, 498
Pyura stolonifera blood plasma, 656

R

Race, Margaret S., see Erich F. Horgan, 460
Rahat, M., and Orit Adar, Effect of symbiotic zooxanthellae and temperature on budding and strobilation in *Cassiopeia andromeda* (Eschscholz), 394
Rall, Jack A., Mechanics and energetics of contraction in striated muscle of the sea scallop, *Placopecton magellanicus*, 489
Reaction-diffusion models of morphogenesis: an application to pattern formation in *Xenopus* retina, 454
Recording from the *Limulus* ventral eye *in situ:* is there a circadian rhythm? 486
Recruitment, of larvae of *Callinectes sapidus*, 402
Regeneration, of crayfish legs, 700, 714
  of fiddler crab legs in magnetic fields, 681
  in gamma-irradiated *Campanularia flexuosa*, 752
Reinschmidt, Dana, see R. Kevin Hunt, 454
  see Robert Tompkins, 455
Reitsma, Carol S., Food choice and palatability in a salt marsh detritivore, *Melampus bidentatus*, 464
Relationship, between diet and growth rate of the grass shrimp *Palaemonetes pugio*, 458
  between dendrite and spine neck diameters in freeze-fractured rat hippocampal formation, 470
  between organic and nitrogen content of marsh sediments and feeding rates in *Uca pugnax*, 460
  between photosystem II and phycobilisomes in red algae and cyanobacteria, 501
  of body colors to environmental light conditions in "poster colored" fishes, 450
Release of cytoskeletal proteins into the perfusate of squid giant axons, 441
Reproduction, coordinated with moulting in terrestrial isotops, 206

  in ascidian *Symplegma reptans*, 219
  see also Fertilization
Reproductive cycles, of the sea urchins of Echinothurioida and Echinoida, 728
Resource partitioning, see competition
Respiration in the polychaete worm *Magelona:* responses to temperature, hypoxia and tentacle ablation, 451
Respiratory metabolism of *Macrobrachium olfersii* (Wiegmann) zoeae during the moulting cycle from eclosion to first ecdysis, 692
Retinotectal patterns and patterns of genetic mosaicism of ploidy chimerae of *Xenopus* eye, 454
Reynolds, Geo. T., Evaluation of low light level intensifier vidicon detectors for microscopy, 473
Rhythm, circadian, 486, 490
  in *Chlamydomonas reinhardii*, affected by caffeine and theophylline, 649
Rifampin as a selective agent for the isolation and enumeration of *Spirochaeta* from salt marsh habitats, 465
mRNA, 476, 478, 498
Rose, Birgit, see Ana Lia Obaid, 489
Rosenberg, Ian, see Sergio Arias-Negrete, 495
  see Eileen Lynch, 496
  see Willy F. Piesens, 497
  see D. Wirth, 498
Ruderman, Joan, see Teresa Tansey, 478
Russell-Hunter, W. D., *et al.*, Lack of respiratory response to temperature acclimation in two littorinid snails, 452
  see David W. Aldridge, 447
  see Robert F. McMahon, 451

S

Saidel, William M., Orthogonal polarization sensitivities of squid photoreceptors: implication for a retinal design, 489
Saito, Takehiko, *et al.*, Circadian rhythm of photoreceptor cells in the *Limulus* lateral eye: further studies, 490
Salama, G., *et al.*, Vanadate inhibits ciliary beating in intact snail salivary glands, 449
Salinity stress, and chitons, 364
  and freshwater clams, 325
  and *Thais haemastoma* osmoregulation, 148
  in *Glycera*, 626
Salzberg, B. M., *et al.*, An optical determination of the resistance in series with the axolemma of *Loligo pealei*, 491
  see G. Salama, 449
Sand dollar, see *Mellita*
Sasner, John J., Jr., see William J. Adelman, Jr., 478
Scanning electron microscopy, of cilia on squid embryo, 102
Schatz, Scott, and Thomas A. Kursar, Photosynthetic activity of fungal infected and noninfected *Laminaria saccharina* Lam., 501
Schimel, J. P., *et al.*, Denitrification potentials in a successional sequence of northern hardwood forest stands, 464
Schistosomiasis immune evasion, 496
Scott, James D., see William R. Kem, 475
Scruggs, Virginia, and David Landowne, The birefringence response of voltage-clamped internally perfused axons, 491
Sea anemone aggressive behavior, 117
Seasonal abundance and distribution of *Crangon franciscorum* and *Palaemon macrodactylus* (Decapoda, Caridae) in the San Francisco Bay-Delta, 117
Sea star, see starfish
Sea urchin, see *Arbacia*, Echinoida, Echinothurioida, *Lytechinus pictus*, *Strongylocentrotus*
Seaweed, aquaculture, 459
  see also algae
Segal, Sheldon, see Mario H. Burgos, 467
  see Michael Coburn, 468
Seitz, Anna W., *et al.*, A developmental analysis of an unusual homoeotic mutation, proboscipedia, in *Drosophila melanogaster*, 454
Selective feeding: by *Neomysis mercedis*, 193
  see also food, feeding
Self/not-self recognition in sea anemones, 117
Senseman, D. M., see G. Salama, 449
Serhan, C., see P. Anderson, 479
Sewage, 465
Shell selection, by hermit crabs, 337
Sher, Alan, see Francis W. Klotz, 496
Sherry, Barbara, see Sara Anderson, 496
Shimada, Junichi, see Gen Matsumoto, 319
Shirai, Hiroko, see Oyewole Adeyomo, 466
  and Yasuaki Yoshimoto, Tension generation in ovarian wall by 1-methyladenine during starfish spawning, 469
Shoaf, Sarah A., *et al.*, Reaction-diffusion models of morphogenesis: an application to pattern formation in *Xenopus* retina, 454
Shrimp, see *Crangon, franciscorum, Macrobrachium, olfersii, Neomysis mercedis, Palaemon macrodactylus, Palaemonetes pugio*
Siegfried, Clifford A., and Mark E. Kopache, Feeding of *Neomysis mercedis* (Holmes), 177
  Seasonal abundance and distribution of *Crangon franciscorum* and *Palaemon macrodactylus* (Decapoda, Caridae) in the San Francisco Bay-Delta, 177
Siliceous teeth, production by *Acartia tonsa*, 349
Silicic acid, deficiency, and effects on tooth formation in *Acartia tonsa* (copepod), 349
  production of media low in, 349
Sinsheimer, Peter, see Michael Coburn, 468
  et al., Toxic effects of vitamin A on sea urchin gametes, 469

Sippewissett Marsh, 456, 458, 459, 461, 463
Sipunculus nudus, mucus secretion, 571
SMITH, FRANCINE R., *et al.*, The interaction of phalloidin with G- and F-actin, 445
SMITH, GEORGE W., Observations on the isolated mitotic apparatus ghost, 445
SMITH, K. M., *et al.*, Chemical conversion of a chlorophyll *a* derivative to a bile pigment, 501
SMITH, STEPHEN J, *et al.*, Arsenazo III reveals long-lasting intracellular calcium transient following the action potential in squid giant presynaptic terminal, 491
  see Milton P. Charlton, 481
  see Robert S. Zucker, 494
Snails, 447, 463, 452, 457
  see *Ilyanassa obsoleta*,
  Littorina littorea
SOCCI, ROBIN, see A. Farmanfarmaian, 458
Sodium channels, 479, 483, 485, 487, 488
Sodium transport in the freshwater Asiatic clam *Corbicula fluminea*, 325
Solid-phase radioimmune assay to detect antibodies in *Entamoeba histolytica*, 495
SOSNOWSKI, RONALD, see Andrew Murray, 476
Specificity in the association between *Hydractinia echinata* and sympatric species of hermit crabs, 337
Sperm, 467, 468, 470, 728
Sperm-oocyte interaction in the surf clam *Spisula solidissima*, 470
Spiders, in Sippewissett Marsh, 456
SPIES, ANNETTE, AND FREDRIC LIPSCHULTZ, Effect of grazing by *Uca pugnax* on the microbial population in salt marsh sediment, 465
Spindles, 443, 445, 446
Spirochaeta, 476
Spirocodon saltatrix, electrical activities, 376
Spisula, 443, 446, 466, 468, 470, 476, 478
SPITSBERGEN, JAN, see James C. Carlisle, 495
Sponge, aggregation factor, 449
  Cliona celata, burrowing and carbonic anhydrase, 135
SPRAY, D. C., see J. H. Stern, 493
Squid, 471, 475, 481–485, 487–492, 494
  axon sodium channels are blocked reversibly by aphantoxin derived from a prokaryotic alga, 478
  development of ciliature pattern on the embryo, 102
  Doryteuthis bleekeri, maintenance in aquaria, 319
Stability of dogfish lens fiber cell membranes, 452
Stages in the life cycle of the digenetic trematode, *Lasiotocus minutus* (Manter, 1931) Thomas, 1959, 497
Starfish, 469, 495
  ultrastructure of coelomocytes, 295
Statistical mechanics of geomagnetic orientation in sediment bacteria, 459
Status of mRNA in eggs and early embryos, 476
STEEL, C. G. H., Mechanisms of coordination between moulting and reproduction in terrestrial isopod Crustacea, 206
STEELE, RODERIC E., AND DANIEL L. GILBERT, Calcium and potassium activities in the hemolymph of the squid, *Loligo pealei*, 492
STEPHENS, PHILIP J. AND C. K. GOVIND, Elimination of synapses from identified lobster motor neurons during development, 492
STERN, J. H., *et al.*, Gap junctions: quantitative comparison of reduction in conductance by H and by Ca ions in an internally perfused preparation, 493
STEUDLER, P. A., see J. P. Schimel, 464
STICKLE, WILLIAM B., see Jane E. Hildreth, 148
Stomachicola, D17
STOPAK, DAVID, AND ROSARIA DE SANTIS, Mitochondrian movements in early development of *Ciona*, 446
STORRIE, BRIAN, see Lucio Cariello, 467
Strobilation, of *Cassiopeia andromeda*, effect of symbionts, 394
Strongylocentrotus skeletal-cell structure, 472
STUART, ANN E., see Kathleen A. French, 483
  see Akimichi Kaneko, 486
Studies, of methanogenic bacteria from intestinal tracts of marine fishes, 457
  of protein synthesis in isolated toadfish hepatocytes, 475
  on reproduction in the compound ascidian, *Symplegma reptans:* Relationship between neural complex and reproduction, 219
STUNKARD, HORACE W. Morphology, life history, and systematic relations of *Tubulovesicula pinguis* (Linton, 1940) Manter, 1947 (Trematoda, Hemiuridae), 737
  Stages in the life cycle of the digenetic trematode, *Lasiotocus minutus* (Manter, 1931) Thomas, 1959, 497
Styela montereyensis, ascidian; orientation, larval behavior, morphology, ecology, 428
Subcellular localization and characterization of islet hormone-degrading enzymes in anglerfish islet tissue, 473
Substitution of calcium by polycations in sponge aggregation factor interaction, 449
SUGIMOTO, KEIJI, AND HIROSHI WATANABE, Studies on reproduction in the compound ascidian *Symplegma reptans:* Relationship between neural complex and reproduction, 219
SULKIN, S. D., W. VAN HUEKELEM, P. KELLY, AND L. VAN HUEKELEM, The behavioral basis of larval recruitment in the crab *Callinectes sapidus* Rathbun: A laboratory investigation of ontogenetic changes in geotaxis and barokinesis, 402
Swelling of squid giant axon during action potentials, 494

Swenson, R. P., Jr., and C. M. Armstrong, External k^+ and Rb^+ retarded closing of potassium channels, 493
Swimbladder, 448
Swinehart, James H., see Clifford J. Hawkins, 656
Symbiosis, of *Cassiopeia andromeda* and algae, 394
  of chemoautotrophic bacteria and marine invertebrates, 456
  hermit crabs and *Hydractinia*, 337
  of *Prochloron* and *Diplosoma virens*, 636
Sympatric, hermit crabs, specificity of association with *Hydractinia echinata*, 337
  mud crabs, electrophoretic variation in, 418
  shrimp *Crangon franciscorum* and *Palaemon macrodactylus*, 177
Symplegma reptans, neural complex and reproduction, 219
Synapse, 481, 491, 492, 494
Synthesis of chlorophyll *a* from δ-aminolevulinate in dark-grown *Cyanidium caldarium* cells, 502
Szaro, Ben G., see R. Kevin Hunt, 454
Szent-Gyorgyi, Albert, see Peter Gascoyne, 474
  see Ronald Pethig, 477

T

Talorchestia longicornis, 455
Tamm, Sidney L. The mechanism of intraplate ciliary synchrony in ctenophores, 446
Tansey, Teresa, and Joan Ruderman, Change in the protein synthetic pattern and mRNA population during *Spisula* embryogenesis, 478
Tasaki, I., and K. Iwasa, Swelling of squid giant axon during action potentials, 494
Tauck, David, see John Moore, 488
Taurine, in *Glycera*, 626
Taxonomy, of *Tubulovesicula pinguis*, 737
Taylor, C. D., see Helmut Brandl, 456
Telzer, Bruce R., and Leah T. Haimo. Binding of dynein to isolated meiotic spindles of the surf clam, *Spisula solidissima*, 446
Temperature acclimation and nitrogen metabolism in littorinid snails, 447
Temperature, effect on budding and strobilation in *Cassiopeia andromeda*, 394
Temperature effects on peak and steady state sodium currents in squid giant axons, 488
Temperature-salinity interaction, and *Thais haemastoma*, 148
Tension generation in ovarian wall by 1-methyladenine during starfish spawning, 469
Teredo navalis (shipworm), effect of competition on larvae in closed culture, 465
Tetrahymena, 471
Thais haemastoma, osmoregulation, 148
Theophylline, effects on *Chlamydomonas reinhardii*, 649
Theory of chemotaxis and the ability of a cell to sense its position and orientation within a tissue, 443
Thermostability of fish hemoglobins, 448
Thrust and drag of bluefish (*Pomatomus saltatrix*) at different buoyancies, speeds, and swimming angles, 450
Tidal water exchanges between Great Sippewissett Salt Marsh and Buzzards Bay, 461
Toadfish (*Opsanus tau*), 475, 477
Tompkins, Robert, *et al.*, Application of a polyploid marker to clonal analysis in *Xenopus* eye, 455
  see R. Kevin Hunt, 454
Toxic effects of vitamin A on sea urchin gametes, 469
Tracey, Gregory A., *et al.*, Effects of closed-culture competitive interactions on growth of *Teredo navalis* larvae, 465
Trematodes, 737, 497
Trench, Robert K., see Charles R. Fisher, Jr., 636
Troll, Walter, see Michael Coburn, 468
  see Peter Sinsheimer, 469
Troxler, R. F., see R. S. Alberte, 498
  see S. B. Brown, 499
  and S. B. Brown, Synthesis of chlorophyll *a* from δ-aminolevulinate in dark-grown *Cyanidium caldarium* cells, 502
  see K. M. Smith, 501
Tsuchiya, Teizo, see Takao Mori, 728
Tubulin genes in parasites, 498
Tullis, Richard E., see William M. Moran, 364
Tumboh-Oeri, Aloys G., and Samuel S. Koide, Sperm-oocyte interaction in the surf clam *Spisula solidissima*, 470
Turner, Katherine, and Timothy A. Lyerla, Electrophoretic variation in sympatric mud crabs from North Inlet, South Carolina, 518
Turner, Ruth D., see Gregory A. Tracey, 465
TV, see video

U

Uca (fiddler crab: feeding rates on sediment, 460
  grazing and microbes, 465
  regeneration and magnetic fields, 681
Ultrastructural characteristics of the nonexpanded and expanded extra-embryonic shell of the horseshoe crab, *Limulus polyphemus*, 582
Ultrastructure, abstracts, 441ff, 470
  of coelomocytes of the sea star *Dermasterias inbricata*, 295
Urn cell comples of *Sipunculus nudus:* A model for study of mucus-stimulating substances, 571
Usem, Michael, see David Hughes, 460

UYEKI, TIMOTHY, AND WENDY WILTSE, The effects of sewage fertilization on benthic macroinvertebrates in salt marsh creeks, 465

V

Vanadate inhibits ciliary beating in intact snail salivary glands, 444
Vanadium, in ascidians, 656, 669
VAN HOLDE, K. E., see W. B. Busa, 442
  see Francine R. Smith, 445
  and M. Brenowitz, A preliminary characterization of the hemocyanin of the giant sea roach, *Bathynomus giganteus*, 478
VAN HUEKELEM, L., see S. D. Sulkin, 402
VAN HUEKELEM, W., see S. D. Sulkin, 402
Video, and firefly mating, 613
  and low light level microscopy, 473
Vision, see photoreceptors, lens
Vitamin A, 469
Vitellogenesis, in terrestrial isopod crustacea, 206
Volume regulation, see osmoregulation, salinity

W

WARGO, JOHN P., The effectiveness of National Park Service policies in protecting barrier island ecosystems within the Cape Cod Natural Seashore, 466
WARNER, JOHN A., AND JAMES F. CASE, The zoogeography and dietary induction of bioluminescence in the midshipman fish, *Porychthys notatus*, 231
WARREN, J. G., see J. P. Schimel, 464
WARREN, M. KIM, see Charles J. Costa, 626
WATANABE, HIROSHI, see Keiji Sugimoto, 219
Water balance, see osmoregulation
WEBER, FREDERICK H. AND E. P. GREENBERG, Rifampin as a selective agent for the isolation and enumeration of *Spirochaeta* from salt marsh habitats, 465
WEIS, JUDITH S., see Paul H. Lee, 681
WEISSMANN, G., see P. Anderson, 479
WERMUTH, JEROME F., Gamma radiation and hydranth longevity in *Campanularia flexuosa:* Age-dependency of dose-response function, 752
WHITEHEAD, DENEENE, see William D. Cohen, 442
WICKHAM, DANIEL E., Aspects of the life history of *Carcinonemertes errans* (Nemertea: Carcinonemertidae), an egg predator of the crab *Cancer magister*, 247
WILLIAMS-ARNOLD, LOIS, see John M. Arnold, 102
WILTSE, WENDY, see Timothy Uyeki, 465
WIRTH, D., Cloning genes of *Leishmania enriettii* in E. coli, 498
  Purification of parasite mRNA, 498
  Tubulin genes in parasites, 498
WITTENBERG, JONATHAN, see Eugene Copeland, 449
WOODWARD, BONNIE L., See Charles B. Miller, 349
WORTHINGTON, C. R., see A. R. Czeto, 482
  see H. Inouye, 485

X

Xenopus (frog): eye, 453, 454
X-ray, study of fish nerves, 485
  study of retinal photoreceptor structure of squid, 482

Y

YOSHIMOTO, YASUAKI, see Hiroko Shirai, 469
YOUNG, CRAIG M., AND LEE F. BRAITHWAITE, Orientation and current-induced flow in the stalked ascidian *Styela montereyensis*, 428
YUDIN, ASHLEY I., RICHARD A. DIENER, W. H. CLARK, JR., AND ERNEST S. CHANG, Mandibular gland of the blue crab, *Callinectes sapidus*, 760
YULO, TERESA, see Seymour Zigman, 452

Z

ZACKS, C. M., see J. P. Schimel, 464
ZIGMAN, SEYMOUR, *et al.*, Stability of dogfish lens fiber cell membranes, 452
Zinc in ascidian plasma, 669
Zoogeography and dietary induction of bioluminescence in the midshipman fish, *Porichthys notatus*, 231
Zostera maria (eel grass), 461, 500
ZUCKER, ROBERT S., *et al.*, Presynaptic calcium currents and facilitated transmitter release in the giant synapse of *Loligo pealei*, 494
  see Milton P. Charlton, 481
  see Stephen J Smith, 491

Continued from Cover Two

high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables, with their headings and footnotes, should be typed on separate sheets, numbered with consecutive Roman numerals, and placed after the Literature Cited. Figure legends should contain enough information to make the figure intelligible separate from the text. Legends should be typed double spaced, with consecutive Arabic numbers, on a separate sheet at the end of the paper. Footnotes should be limited to authors' current addresses, acknowledgments or contribution numbers, and explanation of unusual abbreviations. All such footnotes should appear on the title page. Footnotes are not normally permitted in the body of the text.

4. ***A condensed title*** or running head of no more than 35 letters and spaces should appear at the top of the title page.

5. ***Literature cited.*** In the text, literature should be cited by the Harvard system, with papers by more than two authors cited as Jones *et al.*, 1980. Personal communications and material in preparation or in press should be cited in the text only, with author's initials and institutions, unless the material has been formally accepted and a volume number can be supplied. The list of references following the text sould be headed LITERATURE CITED, and must be typed double spaced on separate pages, conforming in punctuation and arrangement to the style of recent issues of *The Biological Bulletin*. Citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by Biological Abstracts and Chemical Abstracts, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by Biological Abstracts (biosis List of Serials; the most recent issue). Foreign authors, and others who are accustomed to use The World List of Scientific Periodicals, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. useful, since it sets out the World List abbreviations for most biological journals with notes of the USASI abbreviations where these differ. Chemical Abstracts publishes quarterly supplements of additional abbreviations. The following points of reference style for The Biological Bulletin differ from USASI (or modified World List) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in World List *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some Biological Abstracts listings).

H. A few well-known international journals in their preferred forms rather than World List or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond.; Science, N.Y.; Evolution, Lancaster, Pa.*)

6. ***Reprints, charges.*** *The Biological Bulletin* has no page charges. However, authors will be requested to help pay printing charges of manuscripts that are unusually costly due to length or numbers of tables, figures, or formulae. Reprints may be ordered at time of publication and normally will be delivered about two to three months after the issue date. Authors (or delegates or foreign authors) will receive page proofs of articles shortly before publication. They will be charged the current cost of printers' time for corrections to these (other than correction of printers' or editors' errors).

CONTENTS

ERRATUM 503

Invited Article:

ALKON, DANIEL L.
Cellular analysis of a gastropod (*Hermissenda crassicornis*) model of associative learning 505

ALEXANDER, DAVID E., AND JOE GHIOLD
The functional significance of the lunules in the sand dollar, *Mellita quinquiesperforata* 561

BANG, BETSY G., AND FREDERIK B. BANG
The urn cell complex of *Sipunculus nudus:* A model for study of mucus-stimulating substances 571

BANNON, GARY A., AND GEORGE GORDON BROWN
Ultrastructural characteristics of the non-expanded and expanded extra-embryonic shell of the horseshoe crab, *Limulus polyphemus* L. 582

BOUSQUETTE, GEORGE D.
The larval development of *Pinnixa longipes* (Lockington, 1877) (Brachyura: Pinnotheridae), reared in the laboratory 592

BRETOS, MARTA
Age determination in the keyhole limpet *Fissurella crassa* Lamarck (Archaeogastropoda: Fissurellidae), based on shell growth rings 606

CASE, JAMES F.
Courting behavior in a synchronously flashing, aggregative firefly, *Pteroptyx tener* 613

COSTA, CHARLES J., SIDNEY K. PIERCE, AND M. KIM WARREN
The intracellular mechanism of salinity tolerance in polychaetes: Volume regulation by isolated *Glycera dibranchiata* red coelomocytes 626

FISHER, CHARLES R., JR., AND ROBERT K. TRENCH
In vitro carbon fixation by *Prochloron* sp. isolated from *Diplosoma virens* 636

GOODENOUGH, JUDITH E., AND VICTOR G. BRUCE
The effects of caffeine and theophylline on the phototactic rhythm of *Chlamydomonas reinhardii* 649

HAWKINS, CLIFFORD J., PAULINE M. MEREFIELD, DAVID L. PARRY, WILTON R. BIGGS, AND JAMES H. SWINEHART
Comparative study of the blood plasma of the ascidians *Pyura stolonifera* and *Ascidia ceratodes* 656

HAWKINS, CLIFFORD, J., DAVID L. PARRY, AND CRAIG PIERCE
Chemistry of the blood of the ascidian *Podoclavella moluccensis* 669

LEE, PAUL H., AND JUDITH S. WEIS
Effects of magnetic fields on regeneration in fiddler crabs 681

MCNAMARA, JOHN C., GLORIA S. MOREIRA, AND PLINIO S. MOREIRA
Respiratory metabolism of *Macrobrachium olfersii* (Wiegmann) zoeae during the moulting cycle from eclosion to first ecdysis 692

MITTENTHAL, JAY EDWARD
On the form and size of crayfish legs regenerated after grafting 700

MITTENTHAL, JAY EDWARD, MARY C. OLSON, AND GLENNA D. CUMMINGS
Morphology of the closer muscles in normal and homoeotic legs of crayfish 714

MORI, TAKAO, TEIZO TSUCHIYA, AND SHONAN AMEMIYA
Annual gonadal variation in sea urchins of the orders Echinothurioida and Echinoida 728

STUNKARD, HORACE W.
The morphology, life history, and systematic relations of *Tubulovesicula pinguis* (Linton, 1940) Manter, 1947 (Trematoda: Hemiuridae) 737

WERMUTH, JEROME F.
Gamma radiation and hydranth longevity in *Campanularia flexuosa:* Age-dependency of dose-response function 752

YUDIN, ASHLEY I., RICHARD A. DIENER, W. H. CLARK, JR., AND ERNEST S. CHANG
Mandibular gland of the blue crab, *Callinectes sapidus* 760

INDEX TO VOLUME 159 773

NORTHEAST
LIBRARY BINDING CO. INC.
APR 1981